(54) Title: ALLOSTERIC NUCLEIC ACID SENSOR MOLECULES

(57) Abstract: Nucleic acid sensor molecules and methods are provided for the detection and amplification of signaling agents us-
ing enzymatic nucleic acid constructs, including Halfzymes, multicomponent nucleic acid sensor molecules, hammerhead enzymatic
nucleic acid molecules, inozymes, G-cleaver enzymatic nucleic acid molecules, zinzymes, amberzymes and DNAzymes. Also pro-
vided are kits for detection and amplification. The nucleic acid sensor molecules, methods and kits provided herein can be used in
diagnostics, nucleic acid circuits, nucleic acid computers, therapeutics, target validation, target discovery, drug optimization, single
nucleotide polymorphism (SNP) detection, single nucleotide polymorphism (SNP) scoring, and proteome scoring as well as other
uses described herein.

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<u>ALLOSTERIC NUCLEIC ACID SENSOR MOLECULES</u>

This patent application claims priority from Seiwert *et al.,* USSN (TBD), filed
November 1, 2002, which is a continuation-in-part of Seiwert *et al.,* USSN (TBD), filed
October 30, 2002, both entitled "DETECTION OF NUCLEIC ACIDS USING
5   MULTICOMPONENT NUCLEIC ACID SENSOR MOLECULES"; which is a
continuation-in-part of Usman et al., USSN (10/056,761), filed January 23, 2002, which
is a continuation in part of Usman et al., USSN (09/992,160), filed November 5, 2001,
entitled "NUCLEIC ACID SENSOR MOLECULES", which is a continuation-in-part of
Usman et al., USSN (09/877,526) filed June 8, 2001 which is a continuation-in-part of
10   Usman et al. USSN (09/800,594), filed March 6, 2001, entitled "NUCLEIC ACID
SENSOR MOLECULES", which claims priority from Usman et al., USSN (60/187,128),
filed March 6, 2000, entitled "A PROCESS FOR THE DETECTION OF NUCLEIC
ACID USING NUCLEIC ACID CATALYSTS". This patent application also claims
priority from International PCT Application No. PCT/US01/07163 (Publication No. WO
15   01/66721), filed March 6, 2001, entitled "NUCLEIC ACID SENSOR MOLECULES".
These applications are hereby incorporated by reference herein in their entirety including
the drawings.

<u>Field of the Invention</u>

This invention relates to novel molecular sensors, including multicomponent
20   nucleic acid sensors and Halfzymes, that utilize enzymatic nucleic acid constructs whose
activity can be modulated by the presence or absence of various signaling agents. The
present invention further relates to the use of the enzymatic nucleic acid constructs as
molecular sensors capable of modulating the activity, function, or physical properties of
other molecules. The invention also relates to the use of the enzymatic nucleic acid
25   constructs as a diagnostic application, useful in identifying signaling agents in a variety of
applications, for example, in clinical, industrial, environmental, agricultural and/or
research settings. The invention further relates to the use of the nucleic acid sensor

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constructs as a tool to identify the presence of genes and/or gene products which are indicative of a particular genotype and/or phenotype, for example a disease state, infection, or related condition within subjects. In addition, the invention relates to the use of nucleic acid sensor molecules in nucleic acid-based electronics, including nucleic acid-

5   based circuits and computers.

Background of the Invention

The following is a brief description of diagnostic and sensor-based applications for nucleic acids. This summary is provided only for understanding of the invention that follows. This summary is not an admission that all of the work described below is prior

10   art to the claimed invention.

The detection of biomolecules, for example nucleic acids, can be highly beneficial in the diagnosis of diseases or medical disorders. By determining the presence of a specific nucleic acid sequence, investigators can confirm the presence of a virus, bacterium, genetic mutation, and other conditions that can relate to a disease. Assays for

15   nucleic acid sequences can range from simple methods for detection, such as northern blot hybridization using a radiolabeled or fluorescent probe to detect the presence of a nucleic acid molecule, to the use of polymerase chain reaction (PCR) to amplify a small quantity of a specific nucleic acid to the point at which it can be used for detection of the sequence by hybridization techniques. The polymerase chain reaction, uses DNA

20   polymerases to logarithmically amplify the desired sequence (U.S. 4,683,195; U.S. 4,683,202) using prefabricated primers to locate specific sequences. Nucleotide probes can be labeled using dyes, fluorescent, chemiluminescent, radioactive, or enzymatic labels which are commercially available. These probes can be used to detect by hybridization, the expression of a gene or related sequences in cells or tissue samples in

25   which the gene is a normal component, as well as to screen sera or tissue samples from humans suspected of having a disorder arising from infection with an organism, or to detect novel or altered genes as might be found in tumorigenic cells. Nucleic acid primers can also be prepared which, with reverse transcriptase or DNA polymerase and

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PCR, can be used for detection of nucleic acid molecules that are present in very small amounts in tissues or fluids.

PCR utilizes protein enzymes (DNA polymerase) to detect specific nucleotide sequences. PCR has several disadvantages, for example requiring a high degree of

5   technical competence for reliability, high reagent costs, and sensitivity to contamination resulting in false positives.

Several groups to date have completed draft sequences of the entire human genome. To capitalize on this information, an effort to correlate changes in specific mRNA levels with different disease states has been initiated. The synergy of these efforts has been

10   highly successful and there is now a wealth of information relating specific changes in gene expression to disease states. One drawback to the currently available data is that it is not always true that a disease state is reflected by changes in the level of gene expression. Increasingly, post-translational events that control the function of gene products (such as protein processing and protein phosphorylation) have been shown to play important roles

15   in the conversion from a "well" to "diseased" phenotype. Thus, to efficiently use the data generated in the human genome project for the benefit of human health, a profile of disease-specific genomes and proteomes must be generated. Such information will be essential for the generation of treatment outcomes data that link subject and disease characteristics with future treatment events. Therefore, a clear need exists for molecular

20   tools that can generate such disease specific genomes and proteomes or Diagnostic Molecular Profiles that correlate individual cellular and molecular events with disease outcomes profiles. These profiles can then be used to rationally drive treatment policy decisions resulting in better subject care and reductions in health care spending.

A class of enzymes which can be utilized for diagnostic and sensor purposes is

25   enzymatic nucleic acid molecules (Kuwabara *et al.*, 2000, *Curr. Opin. Chem. Bio.*, 4, 669; Porta *et al.*, 1995, *Biochemistry*, 13, 161; Soukup *et al.*, 1999, *TIBTECH*, 17, 469; Marshall *et al.*, 1999, *Nature Struc Biol.*, 6, 992). The enzymatic nature of an enzymatic nucleic acid molecule can be advantageous over other sensor technologies, since the concentration of analyte necessary to generate a detectable response can be lower than

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that required with other sensor systems which can require amplification steps. This advantage reflects the ability of the enzymatic nucleic acid molecule to act enzymatically. Thus, a specific enzymatic nucleic acid molecule is able to amplify a given signal in response to a single recognition event. Such enzymatic nucleic acid-based sensor
5   molecules are often referred to in the art as allosteric ribozymes or allosteric DNAzymes.

In addition, the enzymatic nucleic acid molecule is a highly specific sensor molecule that can be engineered to respond to a variety of different signaling events. The use of *in vitro* selection techniques can be applied to the selection of new enzymatic nucleic acid molecules that are capable of allosteric modulation. Previous work in this
10   area has focused on combining known aptamer and enzymatic nucleic acid molecule sequences (Breaker, International PCT Publication No. WO 98/2714). Later work has revealed bridge sequences that connect the receptor and enzymatic sequence domains together. These bridging sequences function such that binding of a ligand to the receptor domain triggers a conformational change within the bridge, thus modulating
15 · phosphodiester cleavage activity of the adjoining enzymatic sequence (Breaker, International PCT Publication No. WO 00/26226).

George *et al.*, US Patent Nos. 5,834,186 and 5,741,679, describe regulatable RNA molecules whose activity is altered in the presence of a ligand.

Shih *et al.*, US Patent No. 5,589,332, describe a method for the use of ribozymes to
20   detect macromolecules such as proteins and nucleic acid.

Nathan *et al.*, US Patent No 5,871,914, describe a method for detecting the presence of an assayed nucleic acid based on a two component ribozyme system containing a detection ensemble and an RNA amplification ensemble.

Nathan and Ellington, International PCT publication No. WO 00/24931, describe
25   the detection of an analyte by a catalytic nucleic acid sequence which converts a nucleic acid substrate to a catalytic nucleic acid product in the presence of the analyte. The catalytic nucleic acid product is then amplified, by PCR.

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Sullenger *et al.*, International PCT publication No. WO 99/29842, describe nucleic acid mediated RNA tagging and RNA revision.

Usman *et al.*, International PCT Publication No. WO 01/66721, describes nucleic acid sensor molecules.

5   Nathan *et al.*, International PCT Publication No. WO 98/08974, describes specific cofactor-dependent ribozyme constructs.

Summary of the Invention

The present invention relates to nucleic acid-based molecular sensors whose activity can be modulated by the presence or absence of various signaling agents, ligands,

10   and/or target signaling molecules. The invention further relates to a method for the detection of specific target signaling molecules such as nucleic acid molecules, proteins, peptides, antibodies, polysaccharides, lipids, sugars, metals, microbial or cellular metabolites, analytes, pharmaceuticals, and other organic and inorganic molecules using nucleic acid sensor molecules in a variety of analytical settings, including clinical,

15   industrial, veterinary, genomics, environmental, and agricultural applications. The invention further relates to the use of the nucleic acid sensor molecule as molecular sensors capable of modulating the activity, function, or physical properties of other molecules. The present invention also contemplates the use of the nucleic acid sensor molecule constructs as molecular switches, capable of inducing or negating a response in

20   a system, for example in a nucleic acid-based circuit or computer.

The invention further relates to the use of nucleic acid sensor molecules in a diagnostic application to identify the presence of a target signaling molecule such as a gene and/or gene products which are indicative of a particular genotype and/or phenotype, for example, a disease state, infection, or related condition within subjects or subject

25   samples. The invention also relates to a method for the diagnosis of disease states or physiological abnormalities related to the expression of viral, bacterial or cellular RNA and DNA.

The present invention also relates to compounds and methods for the detection of nucleic acid molecules, polynucleotides, and/or oligonucleotides to determine the presence of infectious disease agents in a sample or subject. The invention also relates to compounds and methods for the detection of nucleic acid molecules, polynucleotides,

5   and/or oligonucleotides in a sample or subject as markers or indicators for various diseases and/or conditions in subject. In certain embodiments, the invention relates to novel multicomponent nucleic acid sensor molecules that utilize enzymatic nucleic acid constructs whose activity can be modulated by the presence or absence of signaling agents that include nucleic acids, polynucleotides and/or oligonucleotides associated with

10   a particular infectious agent, disease or condition. The present invention further relates to the use of the multicomponent enzymatic nucleic acid constructs as molecular sensors capable of modulating the activity, function, or physical properties of other nucleic acid molecules useful in detecting nucleic acids, polynucleotides and/or oligonucleotides associated with a particular infectious agent, disease or condition. The invention also

15   relates to the use of the multicomponent enzymatic nucleic acid constructs as diagnostic reagents, useful in identifying such signaling agents in a variety of applications, for example, in screening biological samples or fluids for infectious disease causing agents (e.g., viruses and bacteria) or for screening biological samples or fluids for markers of various diseases or conditions in a subject (e.g., diseases or conditions having a genetic

20   basis).

The invention further relates to the use of multicomponent nucleic acid sensor molecules in a diagnostic application to identify the presence of a target signaling molecule such as a gene and/or gene products which are indicative of a particular genotype and/or phenotype, for example, a disease state, infection, or related condition

25   within subjects or subject samples. The invention also relates to a method for the diagnosis of disease states or physiological abnormalities related to the expression of viral, bacterial or cellular RNA and DNA.

Diagnostic applications of the nucleic acid sensor molecules include the use of the multicomponent nucleic acid sensor molecules for prospective diagnosis of disease,

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prognosis of therapeutic effect and/or dosing of a drug or class of drugs, prognosis and monitoring of disease outcome, monitoring of subject progress as a function of an approved drug or a drug under development, subject surveillance and screening for drug and/or drug treatment. Diagnostic applications include the use of multicomponent nucleic
5   acid sensors for research, development and commercialization of products for the rapid detection of macromolecules, such as mammalian viral nucleic acids for the diagnosis of diseases associated with viruses, prions and viroids in humans and animals.

Diagnostic applications of the nucleic acid sensor molecules include the use of the nucleic acid sensor molecules for prospective diagnosis of disease, prognosis of
10   therapeutic effect and/or dosing of a drug or class of drugs, prognosis and monitoring of disease outcome, monitoring of subject progress as a function of an approved drug or a drug under development, subject surveillance and screening for drug and/or drug treatment. Diagnostic applications include the use of nucleic acid sensors for research, development and commercialization of products for the rapid detection of
15   macromolecules, such as mammalian viral nucleic acids, prions and viroids for the diagnosis of diseases associated with viruses, prions and viroids in humans and animals.

Nucleic acid sensor molecules can also be used in assays to assess the specificity, toxicity and effectiveness of various small molecules, nucleoside analogs, or non-nucleic acid drugs, or doses of a specific small molecules, nucleoside analogs or nucleic acid and
20   non-nucleic acid drugs, against validated targets or biochemical pathways and include the use of nucleic acid sensors in assays involved in high-throughput screening, biochemical assays, including cellular assays, in vivo animal models, clinical trial management, and for mechanistic studies in human clinical studies. The nucleic acid sensor can also be used for the detection of pathogens, biochemicals, for example proteins, organic
25   compounds, or inorganic compounds, in humans, plants, animals or samples therefrom, in connection with environmental testing or detection of biohazards. The use of the nucleic acid sensor molecules in other applications such a functional genomics, target validation and discovery, agriculture or diagnostics, for example the diagnosis of disease, or the prevention or treatment of human or animal disease is also contemplated.

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In one embodiment, the system of the instant invention is an *in vitro* system. The *in vitro* system can be, for example, a sample derived from an organism, mammal, subject, plant, water, beverage, food preparation, or soil or any combination thereof. In another embodiment, the system of the instant invention is an *in vivo* system. The *in vivo* system

5   can be, for example, a bacteria, bacterial cell, fungus, fungal cell, virus, plant, plant cell, mammal, mammalian cell, human or human cell. In another embodiment, the system can be a test sample, for example, a blood sample, serum sample, urine sample, or other tissue sample, cell extract, cell, tissue extract, or entire organism.

In one embodiment, the target signaling molecule of the instant invention is an

10   RNA, DNA, analog of RNA or analog of DNA. In one embodiment, the target signaling molecule of the instant invention is an RNA derived from a bacteria, virus, fungi, plant or mammalian genome.

In one embodiment, the reporter molecule of the instant invention is RNA, DNA, RNA analog, or DNA analog.

15   In one embodiment, the reporter molecule of the instant invention comprises a detectable label selected from the group consisting of chromogenic substrate, fluorescent labels, chemiluminescent labels, and radioactive labels and enzymes. Suitable enzymes include, for example, luciferase, horseradish peroxidase, and alkaline phosphatase.

In another embodiment, the reporter molecule of the instant invention is

20   immobilized on a solid support. Suitable solid supports include silicon-based chips, silicon-based beads, controlled pore glass, polystyrene, cross-linked polystyrene, nitrocellulose, biotin, plastics, metals and polyethylene films.

In one embodiment the sensor component of the nucleic acid sensor molecule is RNA, DNA, analog of RNA or analog of DNA.

25   In another embodiment, the sensor component of the nucleic acid sensor molecule is covalently attached to the nucleic acid sensor molecule by a linker. Suitable linkers include one or more nucleotides, abasic moieties, polyethers, polyamines, polyamides,

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peptides, carbohydrates, lipids, and polyhydrocarbon compounds, and any combination thereof.

In another embodiment, the sensor component of the nucleic acid sensor molecule is not covalently attached to the nucleic acid sensor molecule.

5   In one embodiment, the invention features a nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components, wherein, in response to an interaction of a single stranded RNA (ssRNA) having a single nucleotide polymorphism (SNP) with the nucleic acid sensor molecule in a system, the enzymatic nucleic acid component catalyzes a chemical reaction resulting in a detectable

10   response.

In another embodiment, the invention features a nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components, wherein, in response to an interaction of a single stranded DNA (ssDNA) having a single nucleotide polymorphism (SNP) with the nucleic acid sensor molecule in a system, the

15   enzymatic nucleic acid component catalyzes a chemical reaction resulting in a detectable response.

In yet another embodiment, the invention features a nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components, wherein, in response to an interaction of a peptide with the nucleic acid sensor molecule

20   in a system, the enzymatic nucleic acid component catalyzes a chemical reaction resulting in a detectable response.

In another embodiment, the invention features a nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components, wherein, in response to an interaction of a protein with the nucleic acid sensor molecule

25   in a system, the enzymatic nucleic acid component catalyzes a chemical reaction resulting in a detectable response.

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In one embodiment, the invention features a nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components, wherein, in response to an interaction of a single stranded RNA (ssRNA) with the nucleic acid sensor molecule in a system, the enzymatic nucleic acid component catalyzes a

5   chemical reaction resulting in the cleavage of a predetermined nucleic acid molecule associated with a disease.

In another embodiment, the invention features a nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components, wherein, in response to an interaction of a single stranded DNA (ssDNA) with the nucleic

10   acid sensor molecule in a system, the enzymatic nucleic acid component catalyzes a chemical reaction resulting in the cleavage of a predetermined nucleic acid molecule associated with a disease.

In yet another embodiment, the invention features a nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components,

15   wherein, in response to an interaction of a peptide with the nucleic acid sensor molecule in a system, the enzymatic nucleic acid component catalyzes a chemical reaction resulting in the cleavage of a predetermined nucleic acid molecule associated with a disease.

In another embodiment, the invention features a nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components,

20   wherein, in response to an interaction of a protein with the nucleic acid sensor molecule in a system, the enzymatic nucleic acid component catalyzes a chemical reaction resulting in the cleavage of a predetermined nucleic acid molecule associated with a disease.

In one embodiment, the invention features a nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components,

25   wherein, in response to an interaction of a single stranded RNA (ssRNA) with the nucleic acid sensor molecule in a system, the enzymatic nucleic acid component catalyzes a chemical reaction resulting in ligation of a predetermined nucleic acid molecule to another predetermined nucleic acid molecule.

In another embodiment, the invention features a nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components, wherein, in response to an interaction of a single stranded DNA (ssDNA) with the nucleic acid sensor molecule in a system, the enzymatic nucleic acid component catalyzes a
5   chemical reaction resulting in ligation of a predetermined nucleic acid molecule to another predetermined nucleic acid molecule.

In yet another embodiment, the invention features a nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components, wherein, in response to an interaction of a peptide with the nucleic acid sensor molecule
10   in a system, the enzymatic nucleic acid component catalyzes a chemical reaction resulting in ligation of a predetermined nucleic acid molecule to another predetermined nucleic acid molecule.

In still another embodiment, the invention features a nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components,
15   wherein, in response to an interaction of a protein with the nucleic acid sensor molecule in a system, the enzymatic nucleic acid component catalyzes a chemical reaction resulting in ligation of a predetermined nucleic acid molecule to another predetermined nucleic acid molecule.

In one embodiment, the invention features a method comprising: (a) contacting a
20   nucleic acid sensor molecule of the invention with a system comprising at least one ssRNA having a single nucleotide polymorphism (SNP) under conditions suitable for the enzymatic nucleic acid component of the nucleic acid sensor molecule to catalyze a chemical reaction resulting in a detectable response; and (b) assaying for the detectable response.

25   In another embodiment, the invention features a method comprising: (a) contacting a nucleic acid sensor molecule of the invention with a system comprising at least one ssDNA having a single nucleotide polymorphism (SNP) under conditions suitable for the enzymatic nucleic acid component of the nucleic acid sensor molecule to catalyze a

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chemical reaction resulting in a detectable response; and (b) assaying for the detectable response.

In another embodiment, the invention features a method comprising: (a) contacting a nucleic acid sensor molecule of the invention with a system comprising at least one
5   peptide under conditions suitable for the enzymatic nucleic acid component of the nucleic acid sensor molecule to catalyze a chemical reaction resulting in a detectable response; and (b) assaying for the detectable response.

In yet another embodiment, the invention features a method comprising: (a) contacting a nucleic acid sensor molecule of the invention with a system comprising at
10   least one protein, under conditions suitable for the enzymatic nucleic acid component of the nucleic acid sensor molecule to catalyze a chemical reaction resulting in a detectable response; and (b) assaying for the detectable response.

In one embodiment, the invention features a method comprising contacting a nucleic acid sensor molecule of the invention with a system comprising at least one
15   ssRNA under conditions suitable for the enzymatic nucleic acid component of the nucleic acid sensor molecule to cleave a predetermined nucleic acid molecule.

In another embodiment, the invention features a method comprising the steps of contacting a nucleic acid sensor molecule of the invention with a system comprising at least one ssDNA under conditions suitable for the enzymatic nucleic acid component of
20   the nucleic acid sensor molecule to cleave a predetermined nucleic acid molecule

In yet another embodiment, the invention features a method comprising the steps of contacting a nucleic acid sensor molecule of the invention with a system comprising at least one peptide under conditions suitable for the enzymatic nucleic acid component of the nucleic acid sensor molecule to cleave a predetermined nucleic acid molecule.

25   In another embodiment, the invention features a method comprising the steps of contacting a nucleic acid sensor molecule of the invention with a system comprising at

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least one protein, under conditions suitable for the enzymatic nucleic acid component of the nucleic acid sensor molecule to cleave a predetermined nucleic acid molecule.

In one embodiment, the invention features a method comprising contacting a nucleic acid sensor molecule of the invention with a system comprising at least one 5   ssRNA having a single nucleotide polymorphism (SNP) under conditions suitable for the enzymatic nucleic acid component of the nucleic acid sensor molecule to ligate a predetermined nucleic acid molecule to another predetermined nucleic acid molecule.

In another embodiment, the invention features a method comprising the steps of contacting a nucleic acid sensor molecule of the invention with a system comprising at 10   least one ssDNA having a single nucleotide polymorphism (SNP) under conditions suitable for the enzymatic nucleic acid component of the nucleic acid sensor molecule to ligate a predetermined nucleic acid molecule to another predetermined nucleic acid molecule.

In yet another embodiment, the invention features a method comprising the steps of 15   contacting a nucleic acid sensor molecule of the invention with a system comprising at least one peptide under conditions suitable for the enzymatic nucleic acid component of the nucleic acid sensor molecule to ligate a predetermined nucleic acid molecule to another predetermined nucleic acid molecule.

In another embodiment, the invention features a method comprising the steps of 20   contacting a nucleic acid sensor molecule of the invention with a system comprising at least one protein, under conditions suitable for the enzymatic nucleic acid component of the nucleic acid sensor molecule to ligate a predetermined nucleic acid molecule to another predetermined nucleic acid molecule.

In one embodiment, the invention features a method of using the nucleic acid 25   sensor molecules of the invention to determine the function or validate a predetermined gene target, a predetermined RNA target, a predetermined peptide target, or a predetermined protein target.

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In another embodiment, the invention features a method of using the nucleic acid sensor molecules of the invention to determine a genotype or to characterize single nucleotide polymorphisms (SNPs) in a gene or genome. In another embodiment, the invention features a method of using the nucleic acid sensor molecules of the invention to

5   determine SNP scoring.

In another embodiment, the invention features a method of using the nucleic acid sensor molecules of the invention to determine a proteome, for example a disease specific proteome or treatment specific proteome. In yet another embodiment, the invention features a method of using the nucleic acid sensor molecules of the invention to

10   determine a proteome map or to determine proteome scoring.

In one embodiment, the invention features a method of using the nucleic acid sensor molecules of the invention to determine the dosage of a therapy used in treating a subject, to determine susceptibility of a subject to disease, to determine drug metabolism in a subject, to select a subject for a clinical trail, to determine a choice of therapy in a

15   subject, or to treat a subject.

In another embodiment, the detection of a chemical reaction in a method of the invention is indicative of the presence of the target signaling agent in the system.

In another embodiment, the absence of a chemical reaction in a method of the invention is indicative of the system lacking the target signaling agent.

20   In one embodiment, a system of the invention is an *in vitro* system, for example, a sample derived from an organism, mammal, subject, plant, water, beverage, food preparation, or soil, or any combination thereof.

In another embodiment, a system of the invention is an *in vivo* system, for example, a bacteria, bacterial cell, fungus, fungal cell, virus, plant, plant cell, mammal, mammalian

25   cell, human, or human cell. In another embodiment, the system can be a test sample, for example, a blood sample, serum sample, urine sample, or other tissue sample, cell extract, cell, tissue extract, or entire organism.

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In another embodiment, a component of a nucleic acid sensor molecule of the invention comprises a hammerhead, hairpin, inozyme, G-cleaver, Zinzyme, RNase P EGS nucleic acid, DNAzyme, Amberzyme, or Class I ligase motif.

A chemical reaction of a nucleic sensor molecule of the invention can comprise, for example, cleavage of a phosphodiester internucleotide linkage, ligation of a predetermined nucleic acid molecule to the nucleic acid sensor molecule, ligation of a predetermined nucleic acid molecule to another predetermined nucleic acid molecule, isomerization, phosphorylation of a peptide or protein, dephosphorylation of a peptide or protein, RNA polymerase activity, an increase or decrease in fluorescence, an increase or decrease in enzymatic activity, an increase or decrease in the production of a precipitate, an increase or decrease in chemoluminescence, or an increase or decrease in radioactive emission.

In another embodiment, the invention features a kit comprising a nucleic acid sensor molecule of the invention.

In another embodiment, the invention features an array of nucleic acid sensor molecules comprising a predetermined number of nucleic acid sensor molecules of the invention. In one embodiment, a nucleic acid sensor molecule of the instant invention is attached to a solid surface. Preferably, the surface of the instant invention comprises silicon-based chips, silicon-based beads, controlled pore glass, polystyrene, cross-linked polystyrene, nitrocellulose, biotin, plastics, metals and polyethylene films.

In another embodiment, the invention features a nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components, wherein, in response to an interaction of a Hepatitis C virus (HCV) peptide with the nucleic acid sensor molecule in a system, the enzymatic nucleic acid component catalyzes a chemical reaction resulting in resulting in the cleavage of a predetermined RNA molecule associated with a disease, for example Hepatitis C virus (HCV) RNA.

In yet another embodiment, the invention features a nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components,

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wherein, in response to an interaction of a Hepatitis C virus (HCV) protein, for example a HCV core protein or coat protein, with the nucleic acid sensor molecule in a system, the enzymatic nucleic acid component catalyzes a chemical reaction resulting in resulting in the cleavage of a predetermined RNA molecule associated with a disease, for example

5   HCV RNA.

In one embodiment, a nucleic acid sensor molecule of the invention comprises a sensor component having a sequence derived from the Hepatitis C virus (HCV) 5'-UTR, for example structural domains IIIa-IIIf, I, II or IV.

In another embodiment, the invention features a pharmaceutical composition

10   comprising a nucleic acid sensor molecule in a pharmaceutically acceptable carrier.

In one embodiment, the invention features a method of administering to a cell, for example a mammalian cell or human cell, a nucleic acid sensor molecule of the invention comprising contacting the cell with the nucleic acid sensor molecule under conditions suitable for the administration. The method of administration can be in the presence of a

15   delivery reagent, for example a lipid, cationic lipid, phospholipid, or liposome.

In another embodiment, the invention features a cell, for example a mammalian cell, such as a human cell, plant cell, bacterial cell, or fungal cell, including a nucleic acid sensor molecule of the invention.

In another embodiment, the invention features an expression vector comprising a

20   nucleic acid sequence encoding at least one nucleic acid sensor molecule of the invention in a manner which allows expression of the nucleic acid sensor molecule.

In yet another embodiment, the invention features a mammalian cell, for example a human cell, including an expression vector of the invention.

In one embodiment, an expression vector of the invention further comprises a

25   sequence for a nucleic acid sensor molecule complementary to an RNA having Hepatitis C virus (HCV) sequence.

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In another embodiment, an expression vector of the invention comprises a nucleic acid sequence encoding two or more nucleic acid sensor molecules, which may be the same or different.

5   In another embodiment, a peptide contemplated by the invention is a viral peptide, for example a peptide derived from Hepatitis C virus (HCV), Hepatitis B virus (HBV), Human immunodeficiency virus (HIV), Human papilloma virus (HPV), Human T-cell lymphotroptic virus Type I (HTLV-1), Cytomegalovirus (CMV), Herpes Simplex virus (HSV), Respiratory syncytial virus (RSV), Rhinovirus, West Nile virus (WNV), Hantavirus, Ebola virus, or Encephalovirus.

10   In another embodiment, a protein contemplated by the invention is a viral protein, for example a protein derived from HCV, HBV, HIV, HPV, HTLV-1, CMV, HSV, RSV, Rhinovirus, WNV, Hantavirus, Ebola virus, or Encephalovirus.

In another embodiment, a predetermined RNA of the invention is associated with Hepatitis C virus (HCV) infection.

15   In another embodiment, the method of the instant invention is carried out more than once.

Brief Description of the Drawings

Figure 1 shows a non-limiting example of a "half-zinzyme" nucleic acid sensor molecule that is modulated by the 5'-UTR of the Hepatitis C virus (HCV 5'-UTR). The

20   figure shows both inactive and active forms of the zinzyme sensor molecule (SEQ ID NO. 43). In the presence of the target signaling oligonucleotide (SEQ ID NO. 26) which represents the stem loop IIIB of the HCV 5'-UTR, the zinzyme sensor demonstrates an activity increase of three logs in cleaving the reporter molecule component of the sensor molecule as shown in the graph (+ oligo target) as compared to the sensor molecule in the

25   absence of the target. In the presence of the full length 350 nt HCV 5'-UTR, the zinzyme sensor molecule demonstrates an almost one log increase in activity in cleaving the reporter molecule component of the sensor molecule.

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Figure 2 A-B shows a non-limiting example of half-zinzyme nucleic acid sensor molecule mediated detection of the HCV genome. Figure 2A shows the structure of the 5'-UTR of the HCV genome. The sequence shown is the sequence used as an oligonucleotide target for nucleic acid sensor molecule catalysis. The purine guanosine R/G cleavage site is boxed. Figure 2B shows the results of a half-zinzyme activity assay in which the half-zinzyme was incubated either in the presence or absence of the oligonucleotide target, or in the presence of RNase-H pre-treated HCV 5'-UTR. Half-zinzyme activity is expressed relative to the level observed in the presence of model oligonucleotide. Reactions included a 1:1 molar ratio of target to halfzyme.

Figure 3 A-B shows a non-limiting example of nucleic acid sensor molecule activation by the protein kinase ERK2. Figure 3A shows and assay where the ERK2 nucleic acid sensor molecule (black bars) was incubated either in the presence of ERK2, BSA, or in the absence of any protein and assayed for activity. An enzymatic nucleic acid molecule that lacks the ERK2 sensor region was similarly incubated and assayed for activity (grey bars). Activity is expressed as the rate of substrate RNA cleavage relative to the rate observed in the presence of ERK2. Figure 3B shows a graph of ERK2 concentration dependence in with the concentration of ERK2 was varied as indicated in allozyme reactions. Activity is expressed as the rate of substreate RNA cleavage relative to the maximal rate observed.

Figure 4 A-B shows a non-limiting example of nucleic acid sensor molecule specificity. Figure 4A shows that the ERK2 nucleic acid sensor molecule is MAPK homolog specific. Equal amounts of the mitogen activated protein kinases ERK2, JNK, or P38 were included in reactions containing the ERK2 nucleic acid sensor molecule. Activity is expressed as the rate of substrate RNA cleavage relative to the rate observed in the presence of ERK2. Figure 4B shows the specificity of the ERK2 nucleic acid sensor molecule for activated (phosphorylated) ERK2. An equal amount of unactivated ERK2 (solid circles) or phosphorylated (activated) ERK2 (open circles) was incubated with ERK2 nucleic acid sensor and substrate cleavage was monitored over time. A reaction performed in parallel lacked protein (squares).

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Figure 5 shows a non-limiting example of a nucleic acid sensor ligase molecule of the invention that responds to HCV RNA.

Figure 6 shows a schematic view of the secondary structure of the HCV 5'UTR (Brown *et al.*, 1992, *Nucleic Acids Res.*, 20, 5041-45; Honda *et al.*, 1999, *J. Virol.*, 73, 1165-74). Major structural domains are indicated in bold.

Figure 7 shows the design of a halfzyme used for SNP discrimination. The halfzyme, based on a zinzyme enzymatic nucleic acid motif, (AZB7.1, SEQ ID NO: 50) was designed in a two-part nucleic acid format where one of the parts comprises the reporter molecule covalently linked to a portion of the enzymatic nucleic acid domain of the halfzyme and the second part is provided by a sequence of HBV DNA (HBV 1887, SEQ ID NO: 51). In the presence of the HBV DNA (HBV 1887), the halfzyme assembles into an active configuration to cause cleavage of the reporter molecule. In the absence of HBV DNA (HBV 1887), the halfzyme construct is not expected to form an active conformation and therefore the reporter will not be cleaved. Six different variant sequences of HBV 1887 were tested for cleavage in the presence of the halfzyme (SNPT-2-7, SEQ ID NOS: 52-57). These variant sequences include single nucleotide substitutions at two distinct positions within the cognate DNA sequence. In addition, the corresponding RNA sequence of HBV 1887 (SEQ ID NO: 58) was tested for halfzyme cleavage.

Figure 8 shows results from a halfzyme SNP discrimination study. In the presence of the HBV DNA sequence (HBV 1887; SEQ ID NO 51) and the corresponding RNA version of this sequence (SEQ ID NO: 58) the halfzyme attains active conformation resulting in the cleavage of the reporter sequence. Introduction of single nucleotide variations within the cognate HBV DNA sequence (SEQ ID NOS: 52-57) results in inhibition of halfzyme activity. Similarly, the halfzyme construct used herein can be designed such that the reporter is not covalently linked to a nucleic acid companent of the halfzyme. Cleavage of the reporter by the halfzyme can be detected using a variety of methods, such as using FRET (florescent resonance energy transfer).

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Figure 9 A-D shows a non-limiting example of a nucleic acid sensor molecule activated by a protein kinase. **Figure 9A** shows the design of nucleic acid sensor molecules ERK-HH and ERK-HH/M1. A pre-existing RNA ligand (sensor domain) specific for the unphosphorylated form of ERK2 was fused to a hammerhead catalytic

5   motif through an attenuated stem II structure to produce ERK-HH. Association with substrate RNA (reporter molecule) is prevented if sequences in the 5' substrate binding arm instead pair with sequences in stem II of the hammerhead domain (boxed). ERK-HH/M1 is identical to ERK-HH except that it contains three mutations in the ligand binding domain that prevent ERK2 association. **Figure 9B** shows a graph depicting

10   substrate cleavage over time using a protein-induced nucleic acid sensor molecule. The time course for substrate RNA cleavage promoted by ERK-HH in the presence of unphosphorylated ERK2 is shown as filled circles; the time course for substrate RNA cleavage promoted by ERK-HH in the absence of any protein is shown as open circles. Also shown is a similar analysis of ERK-HH/M1 activity in the presence or absence of

15   unphosphorylated ERK2 (closed and open squares, respectively). The inset depicts a phosphoimage showing conversion of 5'-labeled substrate RNA (S) to product RNA (P) by ERK-HH in the presence (box) or absence (dashed box) of ERK2. **Figure 9C** shows the pH independence of ERK-HH activity. Duplicate reactions containing 500 nM ERK2 were performed and k_{obs} calculated as described in Example 12. Reaction pHs were 6.5,

20   6.8, 7.0, 7.4, 7.7 and 8.1, and buffered with HEPES (pH < 7.0) or TRIS-HCl (pH >/= 7.0). Error is expressed as standard deviation. **Figure 9D** shows a graph depicting substrate cleavage over time using a nucleic acid sensor molecule ERK-HH/M2. ERK-HH/M2 is identical to ERK-HH except that it contains five mutations in the stem I sequence that do not support stem I-stem II interaction. Assays were performed as

25   described in Example 12 in the presence (filled circles) or absence (open circles) of ERK2, using ERK-HH/M2 in place of ERK-HH. The results of **Figure 9D** indicate that protein-dependent nucleic acid sensor activation requires an alternate conformer. The inset shows a chematic depicting nucleic acid sensor-reporter RNA interaction in stem I of ERK-HH/M2. Stem I sequences in the sensor molecule and reporter RNA each carry

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five mutations that maintain sensor molecule-reporter RNA interaction, but do not support stem I-stem II interaction.

Figure 10 is a graph showing the ERK2 concentration dependence of ERK-HH activation. ERK2 was serial diluted so that the final concentration of ERK2 in reactions varied from 500 nM to 70 pM. Activity (k_{obs}) is expressed relative to the activity observed in the absence of ERK2.

Figure 11A-B shows the specificity of nucleic acid sensor molecule activation. Figure 11A shows MAPK subfamily-specific nucleic acid sensor molecule activation. ERK-HH was activated either with 500 nM of rat ERK2, Bovine serum albumin (BSA), rat JNK2 (Sigma Chemical Corp., USA), human p38α (Sigma Chemical Corp., USA), or without any protein as indicated. Activity is expressed as a percentage of the observed activity rate in the presence of 500 nM ERK2. Figure 11B shows phosphorylation state-specific nucleic acid sensor molecule activation. Figure 11B shows a graph depicting substrate cleavage over time using nucleic acid sensor molecule ERK-HH in the presence of unphosphorylated ERK2 (filled circles), phosphorylated ERK2 (filled squares) or in the absence of any protein (open circles). The inset shows low bis-acrylamide PAGE analysis of ppERK2 preparation. K562 cells (ATCC) were maintained at a density of 5 x 10^5 cells/ml in RPMI (Gibco/Life Technologies, U.S.A.) supplemented with 10 % fetal bovine serum (Gemini Bio-Products, Inc, U.S.A.) and 100 U of penicillin and streptomycin per ml. Cycling K562 cells (2 x 10^7) were harvested in kinase extraction buffer, pH 7.4 (KEB: 50 mM β-glycerophosphate, 1.5 mM EGTA, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 2.5 μg/ml pepstatin, 2 mM benzamidine, 1 mM DTT) and lysed with a glass Dounce homogenizer using 20 strokes with pestle A. Cell extracts were clarified by high speed centrifugation and protein concentrations were determined using the Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL). Unphosphorylated ERK2 is indicated by an asterisk (*).

Figure 12A-B shows the detection of ERK2 in mammalian cell lysates. Figure 12A shows an SDS-PAGE of a K562 cell lysate at a final concentration of 0.5 mg/ml

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total protein. Cell lysates were supplemented with exogenous ERK2 at the indicated concentrations (0, 500nm, 400nm, 300nm, 200nm, 100nm, and 50nm). The ERK2 protein is, shown as indicated by the triangle. Protein was visualized by Coomassie staining. Molecular weights of size standards in K_D are indicated (lane 1). **Figure 12B**
5   shows nucleic acid sensor molecule activity. ERK-HH was incubated in 20 % K562 cell lysate (0.5 mg/ml protein final) with a nuclease-stabilized substrate RNA under otherwise standard reaction conditions. Cell lysates were supplemented with exogenous ERK2 at the indicated concentrations (0, 500nm, 400nm, 300nm, 200nm, 100nm, and 50nm). Observed activity rate is expressed relative to the observed activity rate in the presence of
10   500 nM ERK2 in the absence of lysate.

Figure 13A-B shows a solution phase assay using nucleic acid sensor molecules of the invention. **Figure 13A** shows an assay schematic. Activation of ERK-HH by ERK2 promotes cleavage of a substrate RNA (reporter molecule) carrying a quenched fluorescein; the result is relief of quenching of fluorescein emission at 517 nm. A second,
15   constitutive enzymatic nucleic acid molecule promotes cleavage of a substrate RNA (reporter molecule) carrying a quenched cyanine 3 (Cy3); the result is the relief of quenching of Cy3 emission at 568 nm. Normalized signal is derived from the ratio of fluorescein emission to Cy3 emission. **Figure 13B** is a graph showing the results of a duplexed, solution-phase assay. Assays contained the indicated amounts of ERK2 and
20   fluorophore-carrying nuclease stabilized substrate RNAs for ERK-HH and the constitutive enzymatic nucleic acid molecule. Emission at 517 nm (circles) and 568 nm (squares) was measured in the linear phase of the reaction (5.5 hours) using a Hitachi F4500 Fluorescence Spectrophotometer. Second ordinate (right) represents the normalized ERK-HH activation ratio as the ratio of fluorescein to Cy3 signals
25   (diamonds).

Figure 14A-B shows a nucleic acid sensor molecule responsive to phosphorylated ERK2. **Figure 14A** shows a schematic of nucleic acid sensor molecule ppERK-HH, in which a high affinity RNA ligand specific for the phosphorylated form of ERK2 was fused to the hammerhead catalytic motif using the same design elements as in **Figure 9A**.

Figure 14B shows the specificity of ppERK-HH activation as indicated by the relative observed activity rate of ppERK-HH in the presence of 500 nM phosphorylated ERK2 (ppERK2), 500 nM unphosphorylated ERK2, or in the absence of any protein. Activity rate is expressed relative to k_{obs} in the presence of ppERK2.

5   Figure 15 shows a non-limiting example of a generalized multicomponent nucleic acid sensor molecule construct. Multicomponent sensors or "halfzymes" are derived from constitutively active enzymatic nucleic acid molecules (left), by removing a portion of the enzymatic nucleic acid's sequence (center). A target nucleic acid completes the enzymatic nucleic acid (right). The example shown is non-limiting in that additional components 10   (eg. 2, 3, 4, 5 etc.) can be used to modulate the activity of the sensor construct, providing additional stringency requirements or combinations of effector molecules that can be detected by one sensor.

Figure 16 show a non limiting example of a Halfzyme Catalytic 'Platform' comprising a Class I ribozyme ligase (SEQ ID NO: 64), HCV effector nucleic acid (SEQ 15   ID NO: 65), substrate 1 (SEQ ID NO: 66) and substrate 2 (SEQ ID NO: 67). Catalytic activity of the multicomponent sensor directs the attack of the 2' OH of substrate 2 on the alpha-phosphate at the 5' end of substrate 2.

Figure 17 shows a non limiting example of a HCV target signaling agent that can be used to modulate the activity of a sensor molecule of the invention. Stem-loop IIIB of 20   the 5'-UTR is highly conserved. Sequence of the HCV target (or effector) is the most prominently conserved sequence in all HCV isolates.

Figure 18 shows a non limiting example of Directed Molecular Evolution (DME). A DNA sequence library is flanked by defined sequence for PCR. Sequence variants that are inactive (circles) are separated from functional sequences (stars) and amplified. This 25   process is then iterated.

Figure 19 shows a non-limiting example of a Limit of Detection (LOD) by a single turnover HCV-Halfzyme sensor. The 5'-UTR is cleaved by RNase H at sequences

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flanking the HCV effector when base paired to DNA oligonucleotides. The L.O.D. is shown for the 5'- UTR processed by RNase H (squares) and a synthetic oligoribonucleotide (circles).

Figure 20 shows a non-limiting example of the DME procedure used to produce HCV-Halfzyme nucleic acid sensor molecules. The initial sequence library is produced from mixed-sequence overlapping oligonucleotides. Selection is carried out by fractionating molecules that autoligate to substrate 2 in the presence of the HCV effector based on their electrophoretic mobility. The figure inset shows the region of the HCV-Halfzyme effector sequence 'doped' to 30%.

Figure 21 shows the sequence of clone 8/7 HCV-Halfzyme sensor. (see Table II for rate determinations for different clones).

Figure 22 shows kinetic characterization of a single turnover clone 8/7 HCV-Halfzyme sensor molecule. Activity plateaus as a function of pH but not $Mg2+$ concentration.

Figure 23 shows an analysis of RNA-RNA interactions. A shift in electrophoretic mobility of a labeled RNA (in this example HCV effector) by increasing concentrations of an unlabeled RNA (in this example HCV-multicomponent sensor) can be quantified and used to determine affinity.

Figure 24 shows an example of HCV-Halfzyme sensor sequence libraries used in DME-2. Three independently produced libraries based on the clone 8/7 HCV-Halfzyme sensor contained completely random sequence.

Figure 25 shows kinetic characterization of a HCV-Halfzyme sensor library developed through DME-2. HCV-multicomponent sensor library from DME-2 (squares) and original 8/7 HCV-multicomponent sensor (circles) were characterized for the ligation events shown above: autoligation (left) and ligation of the trinucleotide GGA to substrate 2 (right). Sub-stoichiometric amounts of substrate 2 were used to monitor a single cycle of catalysis on the right.

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Figure 26 shows a non-limiting example of Optimized HCV-Halfzymes from DME-2. Clone 38 and clone 21 HCV-Halfzymes obtained from DME-2 have similar sequence inserted into the same position in addition to the sequence changes found in 8/7 HCV-Halfzyme from DME-1.

5   Figure 27 shows an example of Multiple Turnover Configuration 3. The HCV-Halfzyme directs the ligation of the same substrate 2 used in autoligation (single turnover) reactions. Substrate 1 shown is a 23 nucleotide RNA.

Figure 28 shows non limiting example of the optimization of conditions for HCV-Halfzyme L.O.D. determinations. Clone 21 HCV-Halfzyme signal (fraction ligated, A,C) and turnover rate (B,D) were assessed as a function of pH and substrate RNA concentration (A,B), and as a function of substrate RNA concentration and Mg2+ concentration (C,D).

Figure 29 shows the pH dependence of catalyzed and uncatalyzed substrate RNA ligation. Clone 21 HCV-Halfzyme turnover rate in the presence (upper) and absence 15   (lower) of HCV effector.

Figure 30 shows the 2SD Limit of Detection of Configuration 3 constructs. HCV effector oligoribonucleotide was serially diluted so that HCV-Halfzyme reactions contained the indicated number of molecules. The horizontal bar represents background plus two standard deviations.

20   Figure 31 shows an example of Multiple Turnover Configuration 1. The HCV-Halfzyme is truncated by 4 nucleotides relative to the single turnover version of the HCV-Halfzyme. Optimal substrate 2 (substrate 2-4a) forms 3 base pairs with the HCV-Halfzyme. Substrate 1 is a triphosphorylated trinucleotide.

Figure 32 shows sequences of HCV halfzyme sequences dervied from directed 25   molecular evolution (DME) studies.

Detailed Description of the Invention

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The present invention features compounds, compositions, methods, and kits for the detection of specific nucleic acid based target signaling agents indicative of disease causing agents or markers or disease. The target signaling agents comprise nucleic acids, polynucleotides, and/or oligonucleotides. The target signaling agents further comprise effector molecules that modulate the activity of multicomponent nucleic acid sensor molecules by providing a component to the sensor construct that can modulate the activity of the sensor molecule.

In one embodiment, the invention features a multicomponent nucleic acid sensor molecule comprising one or more enzymatic nucleic acid components, wherein, in response to an interaction of one or more effector components with an enzymatic nucleic acid sensor component in a system, the multicomponent nucleic acid sensor molecule catalyzes a chemical reaction involving ligation. In another embodiment, the ligation reaction involves covalent attachment of one reporter molecule (a first substrate) to another reporter molecule (a second substrate). In another embodiment, the ligation reaction results in the formation of a phosphodiester bond. In another embodiment, a first or second substrate comprises a terminal phosphate group. In yet another embodiment, the reporter molecule of the invention comprises one or more polynucleotides.

In another embodiment, the invention features a multicomponent nucleic acid sensor molecule comprising one or more enzymatic nucleic acid components, wherein, in response to an interaction of one or more effector components with an enzymatic nucleic acid sensor component in a system, the multicomponent nucleic acid sensor molecule catalyzes a chemical reaction involving cleavage. In another embodiment, the cleavage reaction involves phosphodiester cleavage. In yet another embodiment, the reporter molecule of the invention comprises one or more polynucleotides.

In one embodiment, the invention features a nucleic acid sensor molecule comprising an enzymatic nucleic acid component and a separate effector component, wherein the enzymatic nucleic acid is assembled from two or more separate nucleic acid molecules, wherein the separate effector component is one of the two ore more separate nucleic acid molecules that make up the enzymatic nucleic acid component

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of the nucleic acid sensor molecule, such the in the presence of the separate effector compeont, the enzymatic nucleci acid component assembles in a form necessary to enable the nucleic acid sensor molecule to catalyze a chemical reaction involving one or more reporter molecules, and wherein the effector and the reporter molecules are 5 separate molecules.

In one embodiment, the chemcial reaction catalyzed by a nucleic acid sensor molecule of the invention is a ligation reaction. In another embodiment, the ligation reaction involves covalent attachment of a first reporter molecule to a second reporter molecule. In another embodiment, the ligation reaction results in the formation of a 10 phosphodiester bond. In yet another embodiment, the first or second reporter molecule independently comprises a terminal phosphate group.

In one embodiment, the chemcial reaction catalyzed by a nucleic acid sensor molecule of the invention is a phosphodiester cleavage reaction.

In another embodiment, a reporter molecule of the invention comprises one or 15 more polynucleotides.

In one embodiment, the enzymatic nucleic acid component of a nucleic acid sensor molecule of the invention is assembled from two separate nucleic acid molecules. In another embodiment, the enzymatic nucleic acid component of a nucleic acid sensor molecule of the invention is assembled from three separate nucleic 20 acid molecules.

In one embodiment, the invention features a method, comprising: (a) contacting one or more enzymatic nucleic acid components of a multicomponent nucleic acid sensor molecule with a system under conditions suitable for one or more effector components that may be present in the system to interact with a enzymatic nucleic acid component of 25 the multicomponent nucleic acid sensor molecule and to catalyze a chemical reaction involving the ligation of at least a portion of a first reporter molecule to at least a portion

of a second reporter molecule; and (b) assaying for the ligation of at least a portion of a first reporter molecule to at least a portion of a second reporter molecule.

In one embodiment, the invention features a method, comprising: (a) contacting a nucleic acid sensor molecule of the invention with a system under conditions suitable for

5   the nucleic acid sensor molecule and to catalyze a chemical reaction on a reporter molecule; and (b) assaying for the chemical reaction on the reporter molecule

In another embodiment, the invention features a method, comprising: (a) contacting one or more enzymatic nucleic acid components of a multicomponent nucleic acid sensor molecule with a system under conditions suitable for one or more effector components

10   that may be present in the system to interact with a enzymatic nucleic acid component of the multicomponent nucleic acid sensor molecule and to catalyze a chemical reaction involving phosphodiester cleavage of a reporter molecule; and (b) assaying for the cleavage reaction.

In one embodiment, a method of the invention further features treating the system

15   under conditions for an effector component of a multicomponent nucleic acid sensor molecule is available to interact with an enzymatic nucleic acid component of a multicomponent nucleic acid sensor molecule of the invention. Such treatment can comprise the use of reagents that cleave RNA or DNA at predetermined sites or alternately cleave RNA or DNA randomly.

20   In one embodiment, the detection of a ligation reaction catalyzed by a nucleic acid sensor molecule of the instant invention is indicative of the presence of the effector component or target nucleic acid molecule in a system.

In another embodiment, the absence of a ligation reaction catalyzed by a nucleic acid sensor molecule of the instant invention is indicative of a system lacking the effector

25   component or target nucleic acid molecule.

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In one embodiment, the detection of a cleavage reaction catalyzed by a nucleic acid sensor molecule of the instant invention is indicative of the presence of the effector component or target nucleic acid molecule in a system.

In another embodiment, the absence of a cleavage reaction catalyzed by a nucleic
5   acid sensor molecule of the instant invention is indicative of a system lacking the effector component or target nucleic acid molecule.

In one embodiment, the system of the instant invention is an *in vitro* system. The *in vitro* system can be, for example, a sample derived from an organism, mammal, subject, plant, water, beverage, food preparation, or soil or any combination thereof. In another
10   embodiment, the system of the instant invention is an *in vivo* system. The *in vivo* system can be, for example, a bacteria, bacterial cell, fungus, fungal cell, virus, plant, plant cell, mammal, mammalian cell, human or human cell. In another embodiment, the system can be a test sample, for example, a blood sample, serum sample, saliva sample, urine sample, or other tissue sample, cell extract, cell, tissue extract, or entire organism.

15. In one embodiment, the effector component of a multicomponent nucleic acid sensor molecule of the instant invention is an RNA, DNA, analog of RNA or analog of DNA. In one embodiment, the effector component of a multicomponent nucleic acid sensor molecule of the instant invention is an RNA or DNA derived from a bacteria, virus, fungi, plant or mammalian genome. In yet another embodiment, the effector
20   component of a multicomponent nucleic acid sensor molecule of the instant invention is a component of a system, sample, or subject.

In one embodiment, a reporter molecule of the instant invention is RNA, DNA, RNA analog, or DNA analog.

In one embodiment, the reporter molecule of the instant invention comprises a
25   detectable label selected from the group consisting of chromogenic substrate, fluorescent labels, chemiluminescent labels, and radioactive labels and enzymes. Suitable enzymes include, for example, luciferase, horseradish peroxidase, and alkaline phosphatase.

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In another embodiment, the reporter molecule of the instant invention is immobilized on a solid support. Suitable solid supports include silicon-based chips, silicon-based beads, controlled pore glass, polystyrene, cross-linked polystyrene, nitrocellulose, biotin, plastics, metals and polyethylene films.

5   In one embodiment an enzymatic nucleic acid component of the nucleic acid sensor molecule is RNA, DNA, analog of RNA or analog of DNA.

In another embodiment, a reporter molecule of the invention is covalently attached by a linker to one or more components of a multicomponent nucleic acid sensor molecule of the invention. Suitable linkers include one or more nucleotides, abasic moieties, 10   polyethers, polyamines, polyamides, peptides, carbohydrates, lipids, and polyhydrocarbon compounds, and any combination thereof.

In another embodiment, a reporter molecule of the invention is not covalently attached a component of a nucleic acid sensor molecule.

In another embodiment, the invention features a kit comprising a nucleic acid 15   sensor molecule of the invention. The kit of the invention can further include any additional reagents, reporter molecules, buffers, excipients, containers and/or devices as required described herein or known in the art, to practice a method of the invention.

In another embodiment, the invention features an array of one or more enzymatic nucleic acid components of a multicomponent nucleic acid sensor molecule of the 20   invention comprising a predetermined number of enzymatic nucleic acid components. In one embodiment, an enzymatic nucleic acid component of the instant invention is attached to a solid surface. The surface can comprise silicon-based chips, silicon-based beads, controlled pore glass, polystyrene, cross-linked polystyrene, nitrocellulose, biotin, plastics, metals and polyethylene films.

25   In one embodiment, an effector component of a multicomponent nucleic acid sensor molecule of the invention comprises a sequence derived from Hepatitis C virus (HCV), Hepatitis B virus (HBV), human immunodeficiency virus (HIV), human

papilloma virus (HPV), poliovirus, West Nile virus (WNV), cytomegalovirus (CMV), Herpes Simplex Virus (HSV), respiratory syncytial virus (RSV), influenza virus, rhinovirus, foot and mouth disease virus, Ebola virus, dengue fever virus, feline leukemia virus (FLV), encephalovirus.

5   In one embodiment, an effector component of a multicomponent nucleic acid sensor molecule of the invention comprises a sequence derived from a disease causing gene, splice variant, or from a small nucleotide polymorphism (SNP). Such sequences can be indicative of cancer, metabolic disorders, and other diseases or conditions having a genetic basis.

10   In one embodiment, an effector component of a multicomponent nucleic acid sensor molecule of the invention comprises a sequence derived from the Hepatitis C virus (HCV) 5'-UTR, for example structural domains IIIa-IIIf, I, II or IV.

       In one embodiment, an effector component of a multicomponent nucleic acid sensor molecule of the invention comprises a sequence derived from a bacterium, such as
15   Corynebacteria, Pneumococci, Streptococci, Staphylococci, enteric bacilli, mycobacteria, spirochetes, chlamydiae. In another embodiment, an effector component of a multicomponent nucleic acid sensor molecule of the invention comprises a sequence derived from bacterial ribosomal RNA.

       In another embodiment, the invention features an expression vector comprising a
20   nucleic acid sequence encoding at least one component of a multicomponent nucleic acid sensor molecule of the invention in a manner which allows expression of the component.

       In yet another embodiment, the invention features a mammalian cell, for example a human cell, including an expression vector of the invention.

       In one embodiment, the invention features a multicomponent nucleic acid sensor
25   molecule that is used to assay the presence of a nucleic acid, polynucleotide, or oligonucleotide in a system or sample, such as a biological system or sample, which is indicative of a disease or condition in a subject, or which is indicative of a disease

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causing agent in the biological system or sample. Non-limiting examples of disease causing agents contemplated by the invention include viral disease causing agents (such as Hepatitis C virus (HCV), Hepatitis B virus (HBV), human immunodeficiency virus (HIV), human papilloma virus (HPV), poliovirus, West Nile virus (WNV),

5   cytomegalovirus (CMV), Herpes Simplex Virus (HSV), respiratory syncytial virus (RSV), influenza virus, rhinovirus, foot and mouth disease virus, Ebola virus, dengue fever virus, feline leukemia virus (FLV), encephalovirus and others) and bacterial disease causing agents (such as Corynebacteria, Pneumococci, Streptococci, Staphylococci, enteric bacilli, mycobacteria, spirochetes, chlamydiae, and others).

10   The nucleic acids, polynucleotides, and oligonucleotides to be detected are generally referred to herein as effector components of the multicomponent nucleic acid sensor molecule. An enzymatic nucleic acid component of a multicomponent nucleic acid sensor molecule of the invention can interact with nucleic acid, polynucleotide, and/or oligonucleotide effector component to perform a ligase reaction, for example

15   between two nucleic acid substrate molecules. Alternately, an enzymatic nucleic acid component of a multicomponent nucleic acid sensor molecule of the invention can interact with nucleic acid, polynucleotide, and/or oligonucleotide effector component to perform a cleavage reaction, for example a phosphodiester cleavage reaction in a reporter molecule. Additionally, the nucleic acid sensor molecules of the invention can detect

20   nucleic acids, polynucleotides, and/or oligonucleotides in biological fluids (eg. blood, urine, saliva) or in fixed, treated tissue as an indication of the presence of disease or infection, and provide sensitive reagents for diagnosis of diseases and infections or in determining the presence of infection disease agents.

In one embodiment, the invention features a multicomponent nucleic acid sensor

25   molecule having specificity for a specific compound comprising a nucleic acid, polynucleotide, and/or oligonucleotide. Such specificity is inherent in the design of the multicomponent nucleic acid sensor molecule in that the effector component of the multicomponent nucleic acid sensor molecule is derived from the target to be detected. Such specific compounds can be associated with a specific disease having a genetic or

infectious basis, for example a disease resulting from a genetic splice variant, or a nucleic acid, polynucleotide, or oligonucleotide specific to a particular genotype as in the case of hereditary diseases and conditions. In another embodiment, the invention features a multicomponent nucleic acid sensor molecule having specificity for a conserved class of

5   nucleic acid sequences associated with a particular infectious agent or disease marker. Such classes of compounds can be associated with disease in a variety of species or can comprise classes of nucleic acid molecules encoding proteins having differing amino acid sequences and/or compositions.

The invention further includes detection methods using the multicomponent nucleic

10   acid sensor molecules of the invention. In one embodiment, the invention provides methods for the detection of nucleic acids, polynucleotides, and oligonucleotides as markers for infectious disease causing agents and diseases or conditions having a genetic basis.

In one embodiment, the invention comprises methods useful in diagnostic and

15   pathogenesis studies of infectious disease causing agents in biological samples and/or subjects, useful for detection, surveillance, treatment, and control of infectious disease causing agents.

In one embodiment, a component of a multicomponent nucleic acid molecule of the invention is a linear nucleic acid molecule. In another embodiment, a component of a

20   multicomponent nucleic acid molecule of the invention is a linear nucleic acid molecule that can optionally form a hairpin, loop, stem-loop, or other secondary structure. In yet another embodiment, a component of a multicomponent nucleic acid molecule of the invention is a circular nucleic acid molecule.

In another embodiment, the effector component of a multicomponent nucleic acid

25   sensor molecule of the invention is a single stranded oligonucleotide. In another embodiment, the effector component of a multicomponent nucleic acid sensor molecule of the invention is a double-stranded oligonucleotide.

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In one embodiment, a component of a multicomponent nucleic acid sensor molecule of the invention comprises an oligonucleotide having between about 20 and about 500 nucleotides. In another embodiment, a component of a multicomponent nucleic acid sensor molecule of the invention comprises an oligonucleotide having

5   between about 40 and about 250 nucleotides. In another embodiment, a component of a multicomponent nucleic acid sensor molecule of the invention comprises an oligonucleotide having between about 50 and about 150 nucleotides.

In one embodiment, an enzymatic nucleic acid component of a multicomponent nucleic acid sensor molecule of the invention comprises an oligonucleotide having

10   between 20 and 500 nucleotides. In another embodiment, an enzymatic nucleic acid component of a multicomponent nucleic acid sensor molecule of the invention comprises an oligonucleotide having between about 40 and about 250 nucleotides. In another embodiment, an enzymatic nucleic acid component of a multicomponent nucleic acid sensor molecule of the invention comprises an oligonucleotide having between about 50

15   and about 150 nucleotides.

In one embodiment, an effector component of a multicomponent nucleic acid sensor molecule of the invention comprises an oligonucleotide having length sufficient to interact with the enzymatic nucleic acid component resulting in modulation of the multicomponent nucleic acid sensor activity. In another embodiment, an effector

20   component of a multicomponent nucleic acid sensor molecule of the invention comprises an oligonucleotide having between about 7 and about 250 nucleotides. In another embodiment, an effector component of a multicomponent nucleic acid sensor molecule of the invention comprises an oligonucleotide having between about 8 and about 150 nucleotides. In another embodiment, an effector component of a multicomponent nucleic

25   acid sensor molecule of the invention comprises an oligonucleotide comprising a full length RNA or DNA, such as a full length RNA transcript, tRNA or fragment thereof, or a full length DNA or fragment thereof.

In one embodiment, a reporter molecule of the invention comprises a nucleic acid molecule having one or more nucleotides. In another embodiment, a reporter molecule of

the invention of the invention comprises an oligonucleotide having between about 3 and about 250 nucleotides. In another embodiment, an effector component of a multicomponent nucleic acid sensor molecule of the invention comprises an oligonucleotide having between about 4 and about 150 nucleotides.

5　　　In one embodiment, an enzymatic nucleic acid component of a multicomponent nucleic acid sensor molecule of the invention comprises an oligonucleotide having any of SEQ ID NOS: 64, 68, 69, 70, 71, 72, 73, 74, or 75.

In another embodiment, an effector component of a multicomponent nucleic acid sensor molecule of the invention comprises an oligonucleotide having SEQ ID NO: 65.

10　　　In yet another embodiment, a reporter molecule of the invention comprises an oligonucleotide having of SEQ ID NOS: 66, 67, or 76-81.

In one embodiment, the detection and/or quantification of target nucleic acids, polynucleotides, and/or oligonucleotides in a method of the invention is accomplished using a variety of methods, including detecting an increase or decrease in fluorescence, an 15 increase or decrease in enzymatic activity, an increase or decrease in the production of a precipitate, an increase or decrease in chemoluminescence, an increase or decrease in chemiluminescence, or likewise a change in UV absorbance, phosphorescence, pH, optical rotation, isomerization, polymerization, temperature, mass, capacitance, resistance, emission of radiation, or colorimetric change.

20　　　In one embodiment, detection and/or quantitation of the presence of target nucleic acids, polynucleotides, and/or oligonucleotides in the above inventive methods can be accomplished using one or more reporter molecules. The reporter molecules can be attached to the enzymatic nucleic acid component or can be free in the sample. In one embodiment, a reporter molecule of the instant invention comprises one or more nucleic 25 acid substrate molecules having a detectable label selected from the group consisting of chromogenic substrate, fluorescent labels, chemiluminescent labels, and radioactive labels and enzymes. Suitable enzymes include, for example, luciferase, horseradish peroxidase, and alkaline phosphatase.

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In another embodiment, the reporter molecule of the instant invention is immobilized on a solid support. Suitable solid supports include silicon-based chips, silicon-based beads, controlled pore glass, polystyrene, cross-linked polystyrene, nitrocellulose, biotin, plastics, metals and polyethylene films.

5   The present invention features compositions and methods for the detection and/or amplification of specific target signaling agents and target signaling molecules in a system using nucleic acid sensor molecules. In one embodiment, the present invention features a nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components wherein, in response to an interaction with a target

10   signaling agent, the enzymatic nucleic acid component catalyzes a chemical reaction in which the activity or physical properties of a reporter molecule is modulated. Preferably, the chemical reaction in which the activity or physical properties of a reporter molecule is modulated results in a detectable response.

In one embodiment, the present invention features a nucleic acid sensor molecule

15   comprising an enzymatic nucleic acid component and one or more sensor components wherein, in response to an interaction of a target signaling agent with the nucleic acid sensor molecule, the enzymatic nucleic acid component catalyses a chemical reaction involving covalent attachment of at least a portion of a reporter molecule.

The chemical reaction in which a reporter molecule is covalently attached to the

20   nucleic acid sensor molecule can be, for example, a ligation, transesterification, phosphorylation, carbon-carbon bond formation, amide bond formation, peptide bond formation, and disulfide bond formation.

In another embodiment, the present invention features a nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor

25   components wherein, in response to an interaction of a target signaling molecule with the nucleic acid sensor molecule, the enzymatic nucleic acid component carries out a chemical reaction that modulates the activity or properties of the reporter molecule. The chemical reaction in which the activity of a reporter molecule is modulated can be, for

example, a phosphorylation, dephosphorylation, isomerization, polymerization, amplification, helicase activity, transesterification, ligation, hydration, hydrolysis, alkylation, dealkylation, halogenation, dehalogenation, esterification, desterification, hydrogenation, dehydrogenation, saponification, desaponification, amination,

5   deamination, acylation, deacylation, glycosylation, deglycosylation, silation, desilation, hydroboration, epoxidation, peroxidation, carboxylation, decarboxylation, substitution, elimination, oxidation, and reduction reaction, or any combination of these reactions.

In one embodiment, the invention features a nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components

10   wherein, in response to an interaction of a target signaling molecule with the nucleic acid sensor molecule, the enzymatic nucleic acid component can carry out a chemical reaction involving isomerization of at least a portion of a reporter molecule.

In another embodiment, the invention features a nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components

15   wherein, in response to an interaction of a target signaling molecule with the nucleic acid sensor molecule, the enzymatic component catalyses a chemical reaction on a non-oligonucleotide-based portion of a reporter molecule selected from the group consisting of phosphorylation and dephosphorylation reactions.

Nucleic acid sensor molecules, including halfzymes of the invention can have a

20   detection signal, such as from a reporter molecule. Examples of reporter molecules include nucleic acid molecules comprising various tags, probes, beacons, fluorophores, chemophores, ionophores, radio-isotopes, photophores, peptides, proteins, enzymes, antibodies, nucleic acids, and enzymatic nucleic acids or a combination thereof. The reporter molecule may optionally be covalently linked to a portion of the nucleic acid

25   sensor molecule.

In another embodiment, the reporter molecule of the instant invention can be a molecular beacon, small molecule, fluorophore, chemophore, ionophore, radio-isotope, photophore, peptide, protein, enzyme, antibodie, nucleic acid, and enzymatic nucleic acid

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or a combination thereof (see, for example, Singh *et al.*, 2000, *Biotech.*, 29, 344; Lizardi *et al.*, US Patent Nos. 5,652,107 and 5,118,801).

Using such reporter molecules and others known in the art, the detectable response of the instant invention can be monitored by, for example, a change in fluorescence, color

5   change, UV absorbance, phosphorescence, pH, optical rotation, isomerization, polymerization, temperature, mass, capacitance, resistance, and emission of radiation.

Detection of the target signaling event via the chemical reaction or the change in activity or physical properties of the reporter molecule can be assayed by methods known in the art. Amplification of the target signaling event via the chemical reaction or the

10   change in activity or physical properties of the reporter molecule can be accomplished by methods known in the art, for example, modulating polymerase activity. Modulation of polymerase activity can increase polymerization in a chemical reaction, for example, a polymerase chain reaction (PCR) system, resulting in amplification of a target signaling molecule or reporter molecule.

15   In any of the above-described inventive methods, the system can be an *in vitro* system. The *in vitro* system can be, for example, a sample derived from an organism, mammal, subject, plant, water, beverage, food preparation, or soil, or any combination thereof. In any of the above-described inventive methods, the enzymatic nucleic acid component of said nucleic acid sensor molecule can be a hammerhead, hairpin, inozyme,

20   G-cleaver, Zinzyme, RNase P EGS nucleic acid, Class I ligase and Amberzyme motif. Also, in any of the above-described inventive methods, the enzymatic nucleic acid component of said nucleic acid sensor molecule can be a DNAzyme.

In any of the above-described methods, the detection of a chemical reaction is indicative of the presence of the target signaling molecule in the system. In any of the

25   above-described methods, the absence of a chemical reaction is indicative of the system lacking the target signaling molecule.

In one embodiment, the reporter molecule of the instant invention is selected from the group consisting of molecular beacons, small molecules, fluorophores, chemophores,

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ionophores, radio-isotopes, photophores, peptides, proteins, enzymes, antibodies, nucleic acids, and enzymatic nucleic acids or a combination thereof (see for example in Singh *et al.*, 2000, *Biotech.*, 29, 344; Lizardi *et al.*, US Patent Nos. 5,652,107 and 5,118,801).

5   Using such reporter molecules and others known in the art, the detectable response of the instant invention can be monitored by, for example, a change in fluorescence, color change, UV absorbance, phosphorescence, pH, optical rotation, isomerization, polymerization, temperature, mass, capacitance, resistance, and emission of radiation.

         Detection of the target signaling event via the chemical reaction or the change in activity or physical properties of the reporter molecule can be assayed by methods known 10   in the art. Amplification of the target signaling event via the chemical reaction or the change in activity or physical properties of the reporter molecule is accomplished by methods known in the art, for example, modulating polymerase activity. Modulation of polymerase activity can increase polymerization in a chemical reaction, for example, a polymerase chain reaction (PCR) system, resulting in amplification of a target signaling 15   molecule or reporter molecule.

         In any of the above-described inventive methods, the system can be an *in vitro* system. The *in vitro* system can be a sample derived from , for example, an organism, mammal, subject, plant, water, beverage, food preparation, or soil, or any combination thereof.

20   In any of the above described methods, the target signaling molecule can be an RNA, DNA, analog of RNA or analog of DNA. Thus, for example, the reporter molecule can be an RNA, DNA, RNA analog, or DNA analog. Also, in any of the described methods, wherein the targeting signaling molecule is an RNA, preferably the RNA is derived from a bacteria (e.g. Corynebacteria, Pneumococci, Streptococci, Staphylococci, 25   enteric bacilli, mycobacteria, spirochetes, chlamydiae), virus (e.g. Hepatitis C virus (HCV), Hepatitis B virus (HBV), human immunodeficiency virus (HIV), human papilloma virus (HPV), poliovirus, West Nile virus (WNV), Human T-cell Lyphotrophic Virus Type 1 (HTLV-1), cytomegalovirus (CMV), Herpes Simplex Virus (HSV),

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respiratory syncytial virus (RSV), influenza virus, rhinovirus, foot and mouth disease virus, ebola virus, dengue fever virus, feline leukemia virus (FLV)), fungi (e.g. genera Aspergillus Penicillium and Cladosporium), plant (e.g. corn, soy, cotton, wheat) or mammalian (e.g. human, mouse, rat, cat, dog, monkey) genome.

5   In another embodiment, the invention features a method of detecting and/or amplifying a target signaling molecule, wherein the target signaling molecule is RNA sequence derived from a virus (e.g. Hepatitis C virus (HCV), Hepatitis B virus (HBV), human immunodeficiency virus (HIV), human papilloma virus (HPV), poliovirus, West Nile virus (WNV), cytomegalovirus (CMV), Herpes Simplex Virus (HSV), respiratory

10   syncytial virus (RSV), influenza virus, rhinovirus, foot and mouth disease virus, ebola virus, dengue fever virus, feline leukemia virus (FLV)), fungi (e.g. genera Aspergillus Penicillium and Cladosporium), plant (e.g. corn, soy, cotton, wheat) or mammalian (e.g. human, mouse, rat, cat, dog, monkey) genome, bacteria (e.g. Corynebacteria, Pneumococci, Streptococci, Staphylococci, enteric bacilli, mycobacteria, spirochetes,

15   chlamydiae), mycoplasma or other infectious disease agent, in a system, where the system is a biological sample from a subject, animal, blood, food material, water, and/or other potential sources for infectious disease agents. The method comprises the steps of (1) contacting the system with the nucleic acid sensor molecule, where the nucleic acid sensor molecule comprises an sensor component and an enzymatic nucleic acid

20   component, under conditions suitable for preferential interaction of the sensor component with the target signaling molecule that can be present in the system; (2) contacting the system with a reporter molecule under conditions suitable for the enzymatic nucleic acid component of the nucleic acid sensor molecule to catalyze a reaction with the reporter molecule; and (3) detecting the target signaling molecule by measuring any reaction

25   catalyzed in (2).

         In another embodiment, the invention features a method of the detecting and/or amplifying a target signaling molecule, wherein the target signaling molecule is RNA sequence derived from a virus (e.g. Hepatitis C virus (HCV), Hepatitis B virus (HBV), human immunodeficiency virus (HIV), human papilloma virus (HPV), poliovirus, West

Nile virus (WNV), cytomegalovirus (CMV), Herpes Simplex Virus (HSV), respiratory syncytial virus (RSV), influenza virus, rhinovirus, foot and mouth disease virus, ebola virus, dengue fever virus, feline leukemia virus (FLV)), fungi (e.g. genera Aspergillus Penicillium and Cladosporium), plant (e.g. corn, soy, cotton, wheat) or mammalian (e.g.

5   human, mouse, rat, cat, dog, monkey) genome, bacteria (e.g. Corynebacteria, Pneumococci, Streptococci, Staphylococci, enteric bacilli, mycobacteria, spirochetes, chlamydiae), mycoplasma or other infectious disease agent, or other potential sources for infectious disease agents. The method comprises the steps of (1) contacting a reporter molecule with a mixture comprising a system and a nucleic acid sensor molecule having

10   an enzymatic nucleic acid component and a sensor component, under conditions suitable for the enzymatic nucleic acid component of the nucleic acid sensor molecule to interact with the reporter molecule to catalyze a reaction; and (2) detecting a target signaling molecule by measuring the reaction catalyzed in (1). If the target signaling molecule is not present in the system, then the enzymatic nucleic acid component will not catalyze a

15   reaction with the reporter molecule and there will not be a signal to measure.

In another embodiment, one or more nucleic acid sensor molecules are attached to a solid support, for example, a silicon-based surface. Each nucleic acid sensor molecule can be attached via one of its termini by a spacer molecule to allow the nucleic acid sensor molecule to adopt the appropriate conformations without hindrance from the underlying

20   solid support. A test mixture is contacted with one or more nucleic acid sensor molecules, and the mixture is contacted with the solid support. Measurement of a signal generated by the nucleic acid sensor molecule in response to interaction with a target signaling molecule at each address of the array reveals the concentration of each target signaling molecule in the test mixture.

25   In any of the above methods, the enzymatic nucleic acid component of said nucleic acid sensor molecule can be a hammerhead, hairpin, inozyme, G-cleaver, Zinzyme, RNase P, EGS nucleic acid, or Amberzyme motif.

In any of the above methods, the enzymatic nucleic acid component of said nucleic acid sensor molecule can be a DNAzyme.

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In any of the above methods, the reporter molecule can comprise a detectable label selected from the group consisting of chromogenic substrate, fluorescent labels, chemiluminescent labels, and radioactive labels.

In any of the above methods, the reporter molecule can be immobilized on a solid
5   support, preferably comprising silicon-based chips, silicon-based beads, controlled pore glass, polystyrene, cross-linked polystyrene, nitrocellulose, biotin, plastics, metals and polyethylene films.

In one embodiment of the inventive method, the sensor component of the nucleic acid sensor molecule is RNA, DNA, analog of RNA or analog of DNA.

10   In another embodiment, the sensor component of the nucleic acid sensor molecule is covalently attached to the nucleic acid sensor molecule by a linker. Suitable linkers include, for example, one or more nucleotides, abasic moiety, polyether, polyamine, polyamide, peptide, carbohydrate, lipid, and polyhydrocarbon compounds, and any combination thereof.

15   In another embodiment, the sensor component of the nucleic acid sensor molecule is not covalently attached to the nucleic acid sensor molecule.

In one embodiment, the nucleic acid sensor molecules of the invention are used to detect target signaling agents involved in human and animal disease, for example viruses, bacteria, proteins, other pathogens and toxins. Examples of viral target signaling agents
20   include but are not limited to Hepatitis C virus (HCV), Hepatitis B virus (HBV), human immunodeficiency virus (HIV), human papilloma virus (HPV), poliovirus, West Nile virus (WNV), cytomegalovirus (CMV), Herpes Simplex Virus (HSV), respiratory syncytial virus (RSV), influenza virus, rhinovirus, foot and mouth disease virus, ebola virus, dengue fever virus, feline leukemia virus (FLV), and others. Examples of bacterial
25   target signaling agents include but are not limited to Corynebacteria, Pneumococci, Streptococci, Staphylococci, enteric bacilli, mycobacteria, spirochetes, chlamydiae, and others.

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Examples of protein target signaling agents include but are not limited to prions, for example CVJ and BSE associated prions, signal transduction proteins, tyrosine kinases, phosphatases, phosphorylases, dephosphorylases, polymerases and others. Examples of other parasite target signaling agents include but are not limited to pathogenic agents 5 related to malaria, lyme disease (Borrelia burgdorferi), sleeping sickness, giardia, and cryptsporidia.

Examples of toxin target signaling agents include but are not limited to lead, mercury, asbestos, pesticides, herbicides, PCBs, and other organic and inorganic compounds.

10 The present invention also provides kits for the detection of particular targets in test mixtures. The kit comprises separate components containing solutions of a nucleic acid sensor molecule specific for a particular target signaling agent, and containing solutions of the appropriate reporter molecules. In some embodiments, the kit comprises a solid support to which is attached the nucleic acid sensor molecule to the particular target. In 15 further embodiments, the kit further comprises a component containing a standardized solution of the target. With this solution, it is possible for the user of the kit to prepare a graph or table of the detectable signal (for example, fluorescence units vs. target concentration); this table or graph is then used to determine the concentration of the target in the test mixture. Devices that automate the manipulation of such kits, perform the 20 repeated function of the kits, combine various steps of kits, or that generate data from the kits are further contemplated by the instant invention.

In one embodiment, the nucleic acid sensor molecules (allozymes) are used to detect the presence of or absence of single stranded RNA (ssRNA) in a system, for example in a blood sample, cell extract, cell, or entire organism. An array of nucleic acid 25 sensor molecules, for example when attached to a surface such as a chip or bead, can be used to detect and profile ssRNA in a system. As such, nucleic acid sensor molecules can be used in the analysis and/or profiling of gene expression *in vitro* or *in vivo*. The information generated by the nucleic acid sensor array can be used in mapping gene expression patterns and genotyping for various purposes, for example in target discovery,

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target validation, drug discovery, determining susceptibility to disease, determining the potential effect of various treatments or therapies, predicting drug metabolism or drug response, selecting candidates for clinical trials, and for managing the treatment of disease in individual subjects.

5   In another embodiment, the nucleic acid sensor molecules (allozymes) are used to detect the presence of or absence of single nucleotide polymorphisms (hereinafter "SNPs") or single stranded DNA (ssDNA) in a system, for example in a blood sample, cell extract, cell, or entire organism. An array of nucleic acid sensor molecules, for example when attached to a surface such as a chip or bead, can be used to detect and 10   profile SNPs or ssDNA in a system. As such, nucleic acid sensor molecules can be used in SNP discovery, detection, and scoring. In a non-limiting example, a plurality of nucleic acid sensor molecules is used to screen a fetus, infant, child or adult for genetic defects based on the SNP profile of the fetus, infant, child or adult. A sample of genetic material is obtained from, for example amniotic fluid, chorionic villus, blood, or hair and is 15   contacted with an array of nucleic acid sensor molecules. The array of nucleic acid sensor molecules comprises a SNP library such that the presence of any predetermined SNP is indicated by the corresponding nucleic acid sensor by measuring the extent of the signal produced when the nucleic acid sensor interacts with the SNP, for example by measuring fluorescence, color change, precipitate deposition, voltage or current. For example, a 20   nucleic acid computer device of the invention can be integrated into the nucleic acid sensor array such that the output of the array is recorded electronically and can be subsequently downloaded into a database. An individual SNP profile, for example, a list of particular SNPs comprising a genotype, is established from the signals generated by the nucleic acid sensor array. As such, treatment of the fetus, infant, child or adult can be 25   initiated before symptoms arise.

In another embodiment, the information generated by the nucleic acid sensor array can be used in genotyping for various purposes, for example in target discovery, target validation, drug discovery, determining susceptibility to disease, determining the potential effect of various treatments or therapies, predicting drug metabolism or drug response,

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selecting candidates for clinical trials, and for managing the treatment of disease in individual subjects.

In another embodiment, the nucleic acid sensor molecules (allozymes) are used to detect the presence of or absence peptides and/or proteins in a system, for example in a blood sample, cell extract, cell, or entire organism. These nucleic acid molecules can be used in place of Elisa or Western Blot analysis, and provide a broader array of criteria to differentiate proteins and peptides *in vivo*. The nucleic acid sensor molecules can be used to differentiate proteins or peptides that differ in sequence, conformation, activation state or phosphorylation state, or by other post-translational modifications. An array of nucleic acid sensor molecules, for example when attached to a surface such as a chip or bead, can be used to detect and profile peptides and/or proteins in a system. As such, nucleic acid sensor molecules can be used in proteome discovery, detection, and scoring. In a non-limiting example, a plurality of nucleic acid sensor molecule is used to screen a fetus, infant, child or adult's proteome. A sample of genetic material is obtained from, for example amniotic fluid, chorionic villus, blood, or hair and is contacted with an array of nucleic acid sensor molecules. The array of nucleic acid sensor molecules comprises a proteome library such that the presence of any predetermined peptide or protein is indicated by the corresponding nucleic acid sensor by measuring the extent of the signal produced when the nucleic acid sensor interacts with the peptide or protein, for example by measuring fluorescence, color change, precipitate deposition, voltage or current. For example, a nucleic acid computer device of the invention can be integrated into the nucleic acid sensor array such that the output of the array is recorded electronically and can be subsequently downloaded into a database. The information generated by the nucleic acid sensor array can be used in diagnostic molecular profiling applications such as protien mapping or profiling for various purposes, for example in target discovery, target validation, drug discovery, determining susceptibility to disease, determining the potential effect of various treatments or therapies, predicting drug metabolism or drug response, selecting candidates for clinical trials, and for managing the treatment of disease in individual subjects.

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In one embodiment, the nucleic acid sensor molecules (allozymes) of the invention are used for in vivo applications, for example *in vivo* ELISA, drug screening, and gene regulation. In vivo ELISA is essentially equivalent to western blot analysis. An allozyme specific to analyte, for example DNA, RNA, protein, small molecule, metabolite etc., can

5   be constitutively expressed along with green fluorescent protein (GFP). The allozyme is designed such that when activated it cleaves GFP mRNA thus inhibiting GFP expression. In the presence of an analyte, the GFP signal would not be observed and in the absence of the analyte, full expression of GFP would be achieved. Thus, by monitoring GFP expression the analyte concentration (e.g. protein expression) can be calculated.

.10   Similarly in vivo drug screening can be achieved using a similar system. This system would give direct IC50 and EC50 values. In one embodiment, nucleic acid sensor molecule of the invention (allozymes) can be used to modulate gene expression and the expression of RNA and protein *in vivo*. These nucleic acid sensor molecules are designed to respond to a signaling agent, for example, a gene, SNP, mutant protein, wild-type

15   protein, overexpressed protein, mutant RNA, wild-type RNA, compounds, metals, polymers, other molecules and/or drugs in a system., which in turn modulates the activity of the nucleic acid sensor molecule. In response to interaction with a predetermined signaling agent, the nucleic acid sensor molecule's activity is activated or inhibited such that the expression of a particular target is selectively down-regulated. The target can

20   comprise a wild-type protein or RNA, mutant protein or RNA, and/or a predetermined cellular component that modulates gene expression or protein activity. In a specific example, nucleic acid sensor molecules that are activated by interaction with an RNA encoding a target protein are used as therapeutic agents in vivo. The presence of RNA encoding the target protein activates the nucleic acid sensor molecule that subsequently

25   cleaves the RNA encoding the target protein, resulting in the inhibition of protein expression. In this manner, cells that express the target protein are selectively targeted for therapeutic activity.

In another non-limiting example, an allozyme can be activated by a predetermined protein, peptide, or mutant polypeptide that causes the allozyme to inhibit the expression

30   of the gene encoding the protein, peptide, or mutant polypeptide, by, for example,

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cleaving RNA encoded by the gene. In this non-limiting example, the allozyme acts as a decoy to inhibit the function of the protein, peptide, or mutant polypeptide and also inhibit the expression of the protein, peptide, or mutant polypeptide once activated by the protein, peptide, or mutant polypeptide.

5   Preferably, a nucleic acid molecule of the instant invention -is between 13 and 500 nucleotides in length. For example, nucleic acid sensor molecules of the invention are preferably between 25 and 300 nucleotides in length, more preferably between 30 and 150 nucleotides in length, e.g., 34, 36, 38, 46, 47, 56, 65, 78, or 136 nucleotides in length. Exemplary DNAzymes of the invention are preferably between 15 and 400 nucleotides in

10   length, more preferably between 25 and 150 nucleotides in length, e.g., 29, 30, 31, or 32 nucleotides in length (see for example Santoro et al., 1998, Biochemistry, 37, 13330-13342; Chartrand et al., 1995, Nucleic Acids Research, 23, 4092-4096). Those skilled in the art will recognize that all that is required is for the nucleic acid molecule to be of length and conformation sufficient and suitable for the nucleic acid molecule to catalyze a

15   reaction contemplated herein. The length of the nucleic acid molecules of the instant invention are not limiting within the general limits stated.

In a preferred embodiment, the invention provides a method for producing a class of nucleic acid–based diagnostic agents that exhibit a high degree of specificity for the target signaling molecule.

20   In additional embodiments, the invention features a method of detecting target signaling molecules or signaling agents in both in vitro and in vivo applications. In vitro diagnostic applications can comprise both solid support based and solution based chip, multichip-array, micro-well plate, and micro-bead derived applications as are commonly used in the art. In vivo diagnostic applications can include but are not limited to cell

25   culture and animal model based applications, comprising differential gene expression arrays, FACS based assays, diagnostic imaging, and others.

By "signaling agent" or "target signaling agent" is meant a chemical or physical entity capable of interacting with a nucleic acid sensor molecule, specifically a sensor

component of a nucleic acid sensor molecule, in a manner that causes the nucleic acid sensor molecule to be active. The interaction of the signaling agent with a nucleic acid sensor molecule may result in modification of the enzymatic nucleic acid component of the nucleic acid sensor molecule via chemical, physical, topological, or conformational

5   changes to the structure of the molecule, such that the activity of the enzymatic nucleic acid component of the nucleic acid sensor molecule is modulated, for example is activated or deactivated. Signaling agents can comprise target signaling molecules such as macromolecules, ligands, small molecules, metals and ions, nucleic acid molecules including but not limited to RNA and DNA or analogs thereof, proteins, peptides,

10   antibodies, polysaccharides, lipids, sugars, microbial or cellular metabolites, pharmaceuticals, and organic and inorganic molecules in a purified or unpurified form, or physical signals including magnetism, temperature, light, sound, shock, pH, capacitance, voltage, and ionic conditions.

       By "enzymatic nucleic acid" is meant a nucleic acid molecule capable of catalyzing

15   (altering the velocity and/or rate of) a variety of reactions including the ability to repeatedly cleave other separate nucleic acid molecules (endonuclease activity) or ligate other separate nucleic acid molecules (ligation activity) in a nucleotide base sequence-specific manner. Additional reactions amenable to nucleic acid sensor molecules include but are not limited to phosphorylation, dephosphorylation, isomerization, helicase

20   activity, polymerization, transesterification, hydration, hydrolysis, alkylation, dealkylation, halogenation, dehalogenation, esterification, desterification, hydrogenation, dehydrogenation, saponification, desaponification, amination, deamination, acylation, deacylation, glycosylation, deglycosylation, silation, desilation, hydroboration, epoxidation, peroxidation, carboxylation, decarboxylation, substitution, elimination,

25   oxidation, and reduction reactions on both small molecules and macromolecules. Such a molecule with endonuclease and/or ligation activity can have complementarity in a substrate binding region to a specified gene target, and also has an enzymatic activity that specifically cleaves and/or ligates RNA or DNA in that target. That is, the nucleic acid molecule with endonuclease and/or ligation activity is able to intramolecularly or

30   intermolecularly cleave and/or ligate RNA or DNA and thereby inactivate or activate a

target RNA or DNA molecule. This complementarity functions to allow sufficient hybridization of the enzymatic RNA molecule to the target RNA or DNA to allow the cleavage/ligation to occur. 100% complementarity is preferred, but complementarity as low as 50-75% can also be useful in this invention. In addition, nucleic acid sensor molecule can perform other reactions, including those mentioned above, selectively on both small molecule and macromolecular substrates, though specific interaction of the nucleic acid sensor molecule sequence with the desired substrate molecule via hydrogen bonding, electrostatic interactions, and Van der Waals interactions. The nucleic acids can be modified at the base, sugar, and/or phosphate groups. The term enzymatic nucleic acid is used interchangeably with phrases such as ribozymes, catalytic RNA, enzymatic RNA, catalytic DNA, catalytic oligonucleotides, nucleozyme, DNAzyme, RNA enzyme, endoribonuclease, endonuclease, minizyme, leadzyme, oligozyme, finderon or DNA enzyme. All of these terminologies describe nucleic acid molecules with enzymatic activity.

There are several different structural motifs of enzymatic nucleic acid molecules that catalyze cleavage/ligations reaction, including but not limited to hammerhead motif, hairpin motif, hepatitis delta virus motif, G-cleaver motif, Amberzyme motif, inozyme motif, and Zinzyme motif.

By "nucleic acid sensor molecule" as used herein is meant a nucleic acid molecule wherein the activity of the nucleic acid sensor molecule is modulated by the presence or absence of an effector molecule or target siganlling agent. Nonlimiting examples of nucleic acid sensor molecules of the invention are described in Usman et al., International PCT Publication No. WO 01/66721 incorporated by reference herein in its entirety including the drawings. In one embodiment, a nucleic acid sensor molecule or "multicomponent nucleic acid sensor molecule" as used herein refers to a nucleic acid sensor molecule assembled from one or more enzymatic nucleic acid domains (also referred to as "enzymatic nucleic acid components" herein) and one or more effector domains (also referred to as "effector components" herein). The multicomponent nucleic acid sensor molecule is active to catalyze a reaction involving a reporter molecule, when

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all the necessary components that make up the enzymatic nucleic acid domain interact with each other in a functional manner to catalyze the reaction. In one embodiment, the enzymatic nucleic acid domain is assembled from two or more separate nucleic acid molecules wherein the enzymatic nucleic acid domain is active only when all the separate

5   nucleic acid molecules interact with each other in a manner necessary for the enzymatic nucleic acid domain to be active. In another embodiment, the enzymatic nucleic acid domain is made up of two separate nucleic acid molecules (also referred to as "Halfzyme" herein). In another embodiment, the enzymatic nucleic acid domain is assembled from two or more separate nucleic acids, wherein one of the separate nucleic acid molecules is

10   the effector component. The reporter molecule can optionally be covalently attached to a portion of one of the nucleic acid sensor molecule components. The nucleic acid sensor molecule construct can be engineered such that the effector component of the multicomponent construct is provided in a sample or system of interest, i.e., in the absence of an appropriate effector component, the enzymatic nucleic acid component is

15. unable to catalyze a reaction involving a reporter molecule. Whereas in the presence of the effector component, the enzymatic nucleic acid component and effector component are able to assemble into an active enzymatic nucleic acid molecule component (see for example Figure 1) such that the nucleic acid sensor molecule is active to catalyze a reaction on a reporter molecule. He reaction catalyzed by the nucleic acid sensor

20   molecule on a substrate does not cause any modification of the effector molecule. The effector and the reporter molecule are separate molecules.

By "effector component" or "effector molecule" or "effector" as used herein is meant any nucleic acid, polynucleotide, or oligonucleotide capable of interacting with an enzymatic nucleic acid component of a multicomponent nucleic acid sensor molecule in a

25   manner that modulates the activity of the multicomponent nucleic acid sensor molecule. In one embodiment, the interaction of the effector component with the enzymatic nucleic acid component can provide a configuration such that the multicomponent nucleic acid sensor molecule is active in the presence of the effector. In another embodiment, the interaction of the effector component with the enzymatic nucleic acid component can also

30   result in modification of the enzymatic nucleic acid component of the multicomponent

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nucleic acid sensor molecule via chemical, physical, topological, or conformational changes to the structure of the molecule, such that the activity of the enzymatic nucleic acid component of the nucleic acid sensor molecule is modulated, for example is activated or deactivated in the presence of the effector component. Effector components

5   of the instant invention can comprise nucleic acid molecules indicative of infectious disease causing agents and/or markers of disease having a genetic basis. In another embodiment, the effector molecule interacts with the enzymatic nucleic acid component in the active site. In another embodiment, the effector molecule interacts with the enzymatic nucleic acid component in the allosteric site or site different from the active

10   site. In another embodiment, the effector molecule makes up the active site or is an essential part of the active site of the enzymatic nucleic acid domain. The terms "effector component" or "effector" can also be referred to herein as "target nucleic acid" in the sense that the effector component can comprise a particular target nucleic acid molecule to be detected by the nucleic acid sensor molecule of the invention in a system, subject or

15   sample. In one embodiment, the target nucleic acid comprises longer sequence than the effector component, for example wherein the effector component results from cleavage or processing of the target nucleic acid in a method of the invention.

By "enzymatic nucleic acid component" is meant a nucleic acid molecule capable of catalyzing (altering the velocity and/or rate of) a variety of reactions including the

20   ability to repeatedly cleave other separate nucleic acid molecules (endonuclease activity) or ligate other separate nucleic acid molecules (ligation activity) in a nucleotide base sequence-specific manner in the presence of an effector component. An enzymatic nucleic acid component with endonuclease and/or ligation activity can have complementarity in a substrate binding region to a specified reporter molecule, and also

25   has an enzymatic activity that specifically cleaves and/or ligates RNA or DNA reporter molecules. That is, the enzymatic nucleic acid component with endonuclease and/or ligation activity is able to intramolecularly or intermolecularly cleave and/or ligate RNA or DNA and thereby inactivate or activate a target RNA or DNA molecule in the presence of an effector component. This complementarity functions to allow sufficient

30   hybridization of the enzymatic nucleic acid component to the reporter molecule to allow

the cleavage/ligation to occur. 100% complementarity is preferred, but complementarity as low as 50-75% can also be useful in this invention.

The nucleic acids components and reporter molecules of the invention can be modified at the base, sugar, and/or phosphate groups.

5   By "substrate binding arm" or "substrate binding domain" or "substrate binding region" is meant that portion or region of a nucleic acid sensor molecule which is able to interact, for example, via complementarity (i.e., able to base-pair with), with a portion of its substrate or reporter. Preferably, such complementarity is 100%, but can be less if desired. For example, as few as 10 bases out of 14 can be base-paired (see for example

10   Werner and Uhlenbeck, 1995, *Nucleic Acids Research*, 23, 2092-2096; Hammann *et al.*, 1999, *Antisense and Nucleic Acid Drug Dev.*, 9, 25-31). That is, these arms contain sequences within a nucleic acid sensor molecule which are intended to bring the nucleic acid sensor molecule and the target signaling molecule, for example RNA, together through complementary base-pairing interactions. The nucleic acid sensor molecule of the

15   invention can have binding arms that are contiguous or non-contiguous and can be of varying lengths. The length of the binding arm(s) are preferably greater than or equal to four nucleotides and of sufficient length to stably interact with the target RNA. Preferably, the binding arm(s) are 12-100 nucleotides in length. More preferably, the binding arms are 14-24 nucleotides in length (see, for example, Werner and Uhlenbeck,

20   *supra;* Hamman *et al., supra;* Hampel *et al.*, EP0360257; Berzal-Herrance *et al.*, 1993, *EMBO J.*, 12, 2567-73). If two binding arms are chosen, the design is such that the length of the binding arms are symmetrical (i.e., each of the binding arms is of the same length; e.g., five and five nucleotides, or six and six nucleotides, or seven and seven nucleotides long) or asymmetrical (i.e., the binding arms are of different length; e.g., six

25   and three nucleotides; three and six nucleotides long; four and five nucleotides long; four and six nucleotides long; four and seven nucleotides long; and the like).

By "enzymatic portion" or "catalytic domain" is meant that portion or region of the nucleic acid sensor molecule essential for catalyzing a chemical reaction, such as cleavage of a nucleic acid substrate.

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By "system" or "sample" is meant material, in a purified or unpurified form, from biological or non-biological sources, including but not limited to human, animal, soil, food, water, or others sources that comprise the effector component to be detected or amplified. As such, nucleic acid sensor molecules of the invention can be used to assay

5   target compounds in biologic and non-biologic systems, such as in human and animal subjects or in samples of unidentified materials outside of a biological system.

The "biological system" or "biological sample" as used herein can be a eukaryotic system or a prokaryotic system, for example a bacterial cell, plant cell or a mammalian cell, or of plant origin, mammalian origin, yeast origin, Drosophila origin, or

10   archebacterial origin.

By "reporter molecule" is meant a molecule, such as a nucleic acid sequence (e.g., RNA or DNA or analogs thereof) or peptides and/or other chemical moieties, able to stably interact with the nucleic acid sensor molecule and function as a substrate for the nucleic acid sensor molecule. The reporter molecule can be covalently linked to the

15   nucleic acid sensor molecule or a portion of one of the components of a halfzyme. The reporter molecule can also contain chemical moieties capable of generating a detectable response, including but not limited to, fluorescent, chromogenic, radioactive, enzymatic and/or chemiluminescent or other detectable labels that can then be detected using standard assays known in the art. The reporter molecule can also act as an intermediate in

20   a chain of events, for example, by acting as an amplicon, inducer, promoter, or inhibitor of other events that can act as second messengers in a system.

In one embodiment, the reporter molecule of the invention is an oligonucleotide primer, template, or probe, which can be used to modulate the amplification of additional nucleic acid sequences, for example, sequences comprising reporter molecules, target

25   signaling molecules, effector molecules, inhibitor molecules, and/or additional nucleic acid sensor molecules of the instant invention.

By "sensor component" or "sensor domain" of the nucleic acid sensor molecule is meant, a molecule such as a nucleic acid sequence (e.g., RNA or DNA or analogs

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thereof), peptide, or other chemical moiety which can interact with one or more regions of
a target signaling agent or more than one target signaling agents, and which interaction
causes the enzymatic nucleic acid component of the nucleic acid sensor molecule to
modulate, such as inhibit or activate, the catalytic activity of the nucleic acid sensor

5   molecule. In the presence of a signaling agent, the ability of the sensor component, for
example, to modulate the catalytic activity of the enzymatic nucleic acid component is
inhibited or diminished. The sensor component can comprise recognition properties
relating to chemical or physical signals capable of modulating the enzymatic nucleic acid
component via chemical or physical changes to the structure of the nucleic acid sensor

10 molecule. The sensor component can be derived from a naturally occurring nucleic acid
protein binding sequence, for example RNAs that bind viral proteins such as HIV trans-
activation response (TAR), HIV nucleocapsid, TFIIA, rev, rex, Ebola VP35, HCV core
proteins, HBV core proteins; RNAs that bind eukaryotic proteins such as protein kinase R
(PKR), ribosomal proteins, RNA polymerases, and ribonucleoproteins. The sensor

15 component can also be derived from a nucleic acid sequence that is obtained through *in
vitro* or *in vivo* selection techniques as are know in the art. Alternately, the sensor
component can be derived from a nucleic acid molecule (aptamer) which is evolved to
bind to a nucleic acid sequence within a target nucleic acid molecule. Such sequences or
"aptamers" can be designed to bind a specific protein, peptide, nucleic acid, co-factor,

20 metabolite, drug, or other small molecule with varying affinity. The sensor component
can be covalently linked to the nucleic acid sensor molecule, or can be non-covalently
associated. A person skilled in the art will recognize that all that is required is that the
sensor component is able to selectively inhibit the activity of the nucleic acid sensor
molecule.

25   "Complementarity" refers to the ability of a nucleic acid to form hydrogen bond(s)
with another RNA sequence by either traditional Watson-Crick or other non-traditional
types. In reference to the nucleic molecules of the present invention, the binding free
energy for a nucleic acid molecule with its target or complementary sequence is sufficient
to allow the relevant function of the nucleic acid to proceed, e.g., enzymatic nucleic acid

30 cleavage, ligation, isomerization, phosphorylation, or dephosphorylation. Determination

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of binding free energies for nucleic acid molecules is well known in the art (see, e.g., Turner et al., 1987, *CSH Symp. Quant. Biol.* LII pp.123-133; Frier *et al.*, 1986, *Proc. Nat. Acad. Sci. USA* 83:9373-9377; Turner *et al.*, 1987, *J. Am. Chem. Soc.* 109:3783-3785). A percent complementarity indicates the percentage of contiguous residues in a nucleic acid

5   molecule which can form hydrogen bonds (e.g., Watson-Crick base pairing) with a second nucleic acid sequence (e.g., 5, 6, 7, 8, 9, 10 out of 10 being 50%, 60%, 70%, 80%, 90%, and 100% complementary). "Perfectly complementary" means that all the contiguous residues of a nucleic acid sequence will hydrogen bond with the same number of contiguous residues in a second nucleic acid sequence.

10   By "alkyl" group is meant a saturated aliphatic hydrocarbon, including straight-chain, branched-chain, and cyclic alkyl groups. Preferably, the alkyl group has 1 to 12 carbons. More preferably it is a lower alkyl of from 1 to 7 carbons, more preferably 1 to 4 carbons. The alkyl group can be substituted or unsubstituted. When substituted the substituted group(s) are preferably, hydroxyl, cyano, alkoxy, $=O$, $=S$, NO_2 or $N(CH_3)_2$,

15   amino, or SH. The term also includes alkenyl groups which are unsaturated hydrocarbon groups containing at least one carbon-carbon double bond, including straight-chain, branched-chain, and cyclic groups. Preferably, the alkenyl group has 1 to 12 carbons. More preferably it is a lower alkenyl of from 1 to 7 carbons, more preferably 1 to 4 carbons. The alkenyl group can be substituted or unsubstituted. When substituted the

20   substituted group(s) can be preferably, hydroxyl, cyano, alkoxy, $=O$, $=S$, NO_2, halogen, $N(CH_3)_2$, amino, or SH. The term "alkyl" also includes alkynyl groups which have an unsaturated hydrocarbon group containing at least one carbon-carbon triple bond, including straight-chain, branched-chain, and cyclic groups. Preferably, the alkynyl group has 1 to 12 carbons. More preferably it is a lower alkynyl of from 1 to 7 carbons, more

25   preferably 1 to 4 carbons. The alkynyl group can be substituted or unsubstituted. When substituted the substituted group(s) is preferably, hydroxyl, cyano, alkoxy, $=O$, $=S$, NO_2 or $N(CH_3)_2$, amino or SH. Such alkyl groups can also include aryl, alkylaryl, carbocyclic aryl, heterocyclic aryl, amide and ester groups. An "aryl" group refers to an aromatic group which has at least one ring having a conjugated p electron system and includes

carbocyclic aryl, heterocyclic aryl and biaryl groups, all of which can be optionally substituted. The preferred substituent(s) of aryl groups are halogen, trihalomethyl, hydroxyl, SH, OH, cyano, alkoxy, alkyl, alkenyl, alkynyl, and amino groups. An "alkylaryl" group refers to an alkyl group (as described above) covalently joined to an aryl group (as described above). Carbocyclic aryl groups are groups wherein the ring atoms on the aromatic ring are all carbon atoms. The carbon atoms are optionally substituted. Heterocyclic aryl groups are groups having from 1 to 3 heteroatoms as ring atoms in the aromatic ring and the remainder of the ring atoms are carbon atoms. Suitable heteroatoms include oxygen, sulfur, and nitrogen, and include furanyl, thienyl, pyridyl, pyrrolyl, N-lower alkyl pyrrolo, pyrimidyl, pyrazinyl, imidazolyl and the like, all optionally substituted. An "amide" refers to an -C(O)-NH-R, where R is either alkyl, aryl, alkylaryl or hydrogen. An "ester" refers to an -C(O)-OR', where R is either alkyl, aryl, alkylaryl or hydrogen.

By "nucleotide" is meant a heterocyclic nitrogenous base in N-glycosidic linkage with a phosphorylated sugar. Nucleotides are recognized in the art to include natural bases (standard), and modified bases well known in the art. Such bases are generally located at the 1' position of a nucleotide sugar moiety. Nucleotides generally comprise a base, sugar and a phosphate group. The nucleotides can be unmodified or modified at the sugar, phosphate and/or base moiety, (also referred to interchangeably as nucleotide analogs, modified nucleotides, non-natural nucleotides, non-standard nucleotides and other; see for example, Usman and McSwiggen, *supra*; Eckstein *et al.*, International PCT Publication No. WO 92/07065; Usman *et al.*, International PCT Publication No. WO 93/15187; Uhlman & Peyman, *supra* all are hereby incorporated by reference herein). There are several examples of modified nucleic acid bases known in the art as summarized by Limbach *et al.*, 1994, *Nucleic Acids Res.* 22, 2183. Some of the non-limiting examples of chemically modified and other natural nucleic acid bases that can be introduced into nucleic acids include, inosine, purine, pyridin-4-one, pyridin-2-one, phenyl, pseudouracil, 2,4,6-trimethoxy benzene, 3-methyl uracil, dihydrouridine, naphthyl, aminophenyl, 5-alkylcytidines (*e.g.*, 5-methylcytidine), 5-alkyluridines (*e.g.*, ribothymidine), 5-halouridine (*e.g.*, 5-bromouridine) or 6-azapyrimidines or 6-

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alkylpyrimidines (e.g. 6-methyluridine), propyne, quesosine, 2-thiouridine, 4-thiouridine, wybutosine, wybutoxosine, 4-acetylcytidine, 5-(carboxyhydroxymethyl)uridine, 5'-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluridine, beta-D-galactosylqueosine, 1-methyladenosine, 1-methylinosine, 2,2-dimethylguanosine, 3-

5   methylcytidine, 2-methyladenosine, 2-methylguanosine, N6-methyladenosine, 7-methylguanosine, 5-methoxyaminomethyl-2-thiouridine, 5-methylaminomethyluridine, 5-methylcarbonylmethyluridine, 5-methyloxyuridine, 5-methyl-2-thiouridine, 2-methylthio-N6-isopentenyladenosine, beta-D-mannosylqueosine, uridine-5-oxyacetic acid, 2-thiocytidine, threonine derivatives and others (Burgin et al., 1996, Biochemistry, 35,

10   14090; Uhlman & Peyman, supra). By "modified bases" in this aspect is meant nucleotide bases other than adenine, guanine, cytosine and uracil at 1' position or their equivalents; such bases can be used at any position, for example, within the catalytic core of an nucleic acid sensor molecule and/or in the substrate-binding regions of the nucleic acid molecule.

15   By "nucleoside" is meant a heterocyclic nitrogenous base in N-glycosidic linkage with a sugar. Nucleosides are recognized in the art to include natural bases (standard), and modified bases well known in the art. Such bases are generally located at the 1' position of a nucleoside sugar moiety. Nucleosides generally comprise a base and sugar group. The nucleosides can be unmodified or modified at the sugar, and/or base moiety,

20   (also referred to interchangeably as nucleoside analogs, modified nucleosides, non-natural nucleosides, non-standard nucleosides and other; see for example, Usman and McSwiggen, supra; Eckstein et al., International PCT Publication No. WO 92/07065; Usman et al., International PCT Publication No. WO 93/15187; Uhlman & Peyman, supra all are hereby incorporated by reference herein). There are several examples of

25   modified nucleic acid bases known in the art as summarized by Limbach et al., 1994, Nucleic Acids Res. 22, 2183. Some of the non-limiting examples of chemically modified and other natural nucleic acid bases that can be introduced into nucleic acids include, inosine, purine, pyridin-4-one, pyridin-2-one, phenyl, pseudouracil, 2,4,6-trimethoxy benzene, 3-methyl uracil, dihydrouridine, naphthyl, aminophenyl, 5-alkylcytidines (e.g.,

30   5-methylcytidine), 5-alkyluridines (e.g., ribothymidine), 5-halouridine (e.g.,

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5-bromouridine) or 6-azapyrimidines or 6-alkylpyrimidines (e.g. 6-methyluridine),
propyne, quesosine, 2-thiouridine, 4-thiouridine, wybutosine, wybutoxosine, 4-
acetylcytidine, 5-(carboxyhydroxymethyl)uridine, 5'-carboxymethylaminomethyl-2-
thiouridine, 5-carboxymethylaminomethyluridine, beta-D-galactosylqueosine, 1-
methyladenosine, 1-methylinosine, 2,2-dimethylguanosine, 3-methylcytidine, 2-
methyladenosine, 2-methylguanosine, N6-methyladenosine, 7-methylguanosine, 5-
methoxyaminomethyl-2-thiouridine, 5-methylaminomethyluridine, 5-
methylcarbonylmethyluridine, 5-methyloxyuridine, 5-methyl-2-thiouridine, 2-methylthio-
N6-isopentenyladenosine, beta-D-mannosylqueosine, uridine-5-oxyacetic acid, 2-
thiocytidine, threonine derivatives and others (Burgin et al., 1996, Biochemistry, 35,
14090; Uhlman & Peyman, supra). By "modified bases" in this aspect is meant
nucleoside bases other than adenine, guanine, cytosine and uracil at 1' position or their
equivalents; such bases can be used at any position, for example, within the catalytic core
of an nucleic acid sensor molecule and/or in the substrate-binding regions of the nucleic
acid molecule.

By "unmodified nucleotide" is meant a nucleotide with one of the bases adenine,
cytosine, guanine, thymine, uracil joined to the 1' carbon of beta-D-ribo-furanose.

By "modified nucleotide" is meant a nucleotide that contains a modification in the
chemical structure of an unmodified nucleotide base, sugar and/or phosphate.

By "unmodified nucleoside" is meant a nucleoside with one of the bases adenine,
cytosine, guanine, thymine, uracil joined to the 1' carbon of beta-D-ribo-furanose.

By "modified nucleoside" is meant a nucleotide that contains a modification in the
chemical structure of an unmodified nucleoside base or sugar.

By "class I ligase" as used herein in meant an enzymatic nucleic acid molecule as
generally described in Ekland et al., 1995, Science, 269, 364-370.

By "sufficient length" is meant an oligonucleotide of length sufficient to provide
the intended function (such as binding) under the expected condition. For example, a

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binding arm of the enzymatic nucleic acid component of the nucleic acid sensor molecule should be of "sufficient length" to provide stable binding to the reporter molecule under the expected reaction conditions and environment to catalyze a reaction. In a further example, the sensor domain of the nucleic acid sensor molecule should be of sufficient
5   length to interact with a target nucleic acid molecule in a manner that would cause the nucleic acid sensor to be active.

By "stably interact" is meant interaction of the oligonucleotides with target nucleic acid (e.g., by forming hydrogen bonds with complementary nucleotides in the target under physiological conditions) that is sufficient to the intended purpose (e.g., cleavage of
10   target RNA by an enzyme).

By "nucleic acid molecule" as used herein is meant a molecule comprising nucleotides. The nucleic acid can be single, double, or multiple stranded and can comprise modified or unmodified nucleotides or non-nucleotides or various mixtures and combinations thereof. Nucleic acid molecules shall include oligonucleotides, ribozymes,
15   DNAzymes, templates, and primers.

By "oligonucleotide" or "polynucleotide" is meant a nucleic acid molecule comprising a stretch of three or more nucleotides.

In a preferred embodiment the linker region, when present in the nucleic acid sensor molecule and/or reporter molecule is further comprised of nucleotide, non-
20   nucleotide chemical moieties or combinations thereof. Non-limiting examples of non-nucleotide chemical moieties can include ester, anhydride, amide, nitrile, and/or phosphate groups.

In another embodiment, the non-nucleotide linker is as defined herein. The term "non-nucleotide" as used herein include either abasic nucleotide, polyether, polyamine,
25   polyamide, peptide, carbohydrate, lipid, or polyhydrocarbon compounds. Specific examples include those described by Seela and Kaiser, *Nucleic Acids Res.* 1990, *18*:6353 and *Nucleic Acids Res.* 1987, *15*:3113; Cload and Schepartz, *J. Am. Chem. Soc.* 1991, *113*:6324; Richardson and Schepartz, *J. Am. Chem. Soc.* 1991, *113*:5109; Ma et al.,

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Nucleic Acids Res. 1993, *21*:2585 and *Biochemistry* 1993, *32*:1751; Durand et al., *Nucleic Acids Res.* 1990, *18*:6353; McCurdy *et al., Nucleosides & Nucleotides* 1991, *10*:287; Jschke et al., *Tetrahedron Lett.* 1993, *34*:301; Ono *et al., Biochemistry* 1991, *30*:9914; Arnold *et al.,* International Publication No. WO89/02439; Usman *et al.,* International

5   Publication No. WO 95/06731; Dudycz *et al.,* International Publication No. WO 95/11910 and Ferentz and Verdine, *J. Am. Chem. Soc.* 1991, *113*:4000, all hereby incorporated by reference herein. Thus, in a preferred embodiment, the invention features an nucleic acid sensor molecule of the invention having one or more non-nucleotide moieties, and having enzymatic activity to perform a chemical reaction, for example to

10   cleave an RNA or DNA molecule.

By "cap structure" is meant chemical modifications which have been incorporated at either terminus of the oligonucleotide (see for example Wincott *et al.,* WO 97/26270, incorporated by reference herein). These terminal modifications protect the nucleic acid molecule from exonuclease degradation, and can help in delivery and/or localization

15   within a cell. The cap can be present at the 5'-terminus (5'-cap) or at the 3'-terminus (3'-cap) or can be present on both termini. In non-limiting examples: the 5'-cap is selected from the group comprising inverted abasic residue (moiety), 4',5'-methylene nucleotide; 1-(beta-D-erythrofuranosyl) nucleotide, 4'-thio nucleotide, carbocyclic nucleotide; 1,5-anhydrohexitol nucleotide; L-nucleotides; alpha-nucleotides; modified base nucleotide;

20   phosphorodithioate linkage; *threo*-pentofuranosyl nucleotide; acyclic 3',4'-seco nucleotide; acyclic 3,4-dihydroxybutyl nucleotide; acyclic 3,5-dihydroxypentyl nucleotide, 3'-3'-inverted nucleotide moiety; 3'-3'-inverted abasic moiety; 3'-2'-inverted nucleotide moiety; 3'-2'-inverted abasic moiety; 1,4-butanediol phosphate; 3'-phosphoramidate; hexylphosphate; aminohexyl phosphate; 3'-phosphate; 3'-

25   phosphorothioate; phosphorodithioate; or bridging or non-bridging methylphosphonate moiety (for more details see Wincott *et al.,* International PCT publication No. WO 97/26270, incorporated by reference herein). In yet another preferred embodiment the 3'-cap is selected from a group comprising, 4',5'-methylene nucleotide; 1-(beta-D-erythrofuranosyl) nucleotide; 4'-thio nucleotide, carbocyclic nucleotide; 5'-amino-alkyl

30   phosphate; 1,3-diamino-2-propyl phosphate, 3-aminopropyl phosphate; 6-aminohexyl

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phosphate; 1,2-aminododecyl phosphate; hydroxypropyl phosphate; 1,5-anhydrohexitol nucleotide; L-nucleotide; alpha-nucleotide; modified base nucleotide; phosphorodithioate; *threo*-pentofuranosyl nucleotide; acyclic 3',4'-seco nucleotide; 3,4-dihydroxybutyl nucleotide; 3,5-dihydroxypentyl nucleotide, 5'-5'-inverted nucleotide moiety; 5'-5'-inverted abasic moiety; 5'-phosphoramidate; 5'-phosphorothioate; 1,4-butanediol phosphate; 5'-amino; bridging and/or non-bridging 5'-phosphoramidate, phosphorothioate and/or phosphorodithioate, bridging or non bridging methylphosphonate and 5'-mercapto moieties (for more details see Beaucage and Iyer, 1993, *Tetrahedron* 49, 1925; incorporated by reference herein).

By "abasic" or "abasic nucleotide" is meant sugar moieties lacking a base or having other chemical groups in place of a base at the 1' position, (for more details see Wincott *et al.*, International PCT publication No. WO 97/26270).

The term "non-nucleotide" refers to any group or compound which can be incorporated into a nucleic acid chain in the place of one or more nucleotide units, including either sugar and/or phosphate substitutions, and allows the remaining bases to exhibit their enzymatic activity. The group or compound is abasic in that it does not contain a commonly recognized nucleotide base, such as adenine, guanine, cytosine, uracil or thymine. The terms "abasic" or "abasic nucleotide" are meant to include sugar moieties lacking a base or having other chemical groups in place of a base at the 1' position, (for more details see Wincott *et al.*, International PCT publication No. WO 97/26270).

By "RNA" is meant a molecule comprising at least one ribonucleotide residue. By "ribonucleotide" or "2'-OH" is meant a nucleotide with a hydroxyl group at the 2' position of a β-D-ribo-furanose moiety.

By "subject" is meant an organism, which is a donor or recipient of explanted cells or the cells themselves. "Subject" also refers to an organism to which the nucleic acid molecules of the invention can be administered. Preferably, a subject is a mammal or mammalian cells. More preferably, a subject is a human or human cells.

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By "enhanced enzymatic activity" is meant to include activity measured in cells and/or *in vivo* where the activity is a reflection of both the catalytic activity and the stability of the nucleic acid molecules of the invention. In this invention, the product of these properties can be increased *in vivo* compared to an all RNA enzymatic nucleic acid

5   or all DNA enzyme. In some cases, the individual catalytic activity or stability of the nucleic acid molecule can be decreased (i.e., less than ten-fold), but the overall activity of the nucleic acid molecule is enhanced, in vivo.

By "nucleic acid circuit" or "nucleic acid-based circuit" is meant an electronic circuit comprising one or more nucleic acids or oligonucleotides.

10   By "nucleic acid computer" or "nucleic acid-based computer" is meant a computing device or system comprising one or more nucleic acids or oligonucleotides. The nucleic acid computer can be used to interface biological systems, control other devices, or can be utilized to solve problems and/or manipulate data. Furthermore, the nucleic acid computer may comprise nucleic acid circuits.

15   By "halfzyme" is meant an enzymatic nucleic acid molecule assembled from two or more nucleic acid components. The enzymatic nucleic acid in the halfzyme configuration is active to catalyze a reaction involving a reporter molecule, when all the necessary components that make up the enzymatic nucleic acid interact with each other. The reporter molecule may optionally be covalently attached to a portion of one of the

20   halfzyme components. The halfzyme construct can be engineered such that an essential nucleic acid component of the enzymatic nucleic acid is provided by a target signaling agent of interest, i.e., in the absence of an appropriate target signaling agent the halfzyme construct is unable to catalyze a reaction involving a reporter molecule and in the presence of the target signaling agent, the halze construct is able to assemble into an

25   active enzymatic nucleic acid molecule (see for example **Figures 1 and 15**).

By "predetermined RNA molecule" is meant a particular RNA molecule of known sequence, such as a viral RNA, messenger RNA, transfer RNA, ribosomal RNA etc.

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By "system" is meant a group of substances or components that can be collectively combined or identified. A system can comprise a biological system, for example an organism, cell, or components, extracts, and samples thereof. A system can further comprise an experimental or artificial system, where various substances or components

5 are intentionally combined together.

By "detectable response" is meant a chemical or physical property that can be measured, including, but not limited to changes in temperature, pH, frequency, charge, capacitance, or changes in fluorescent, chromogenic, radioactive, enzymatic and/or chemiluminescent levels or properties that can then be detected using standard methods

10 known in the art.

By "single stranded RNA" (ssRNA) is meant a naturally occurring or synthetic ribonucleic acid molecule comprising a linear single strand, for example a ssRNA can be a messenger RNA (mRNA), transfer RNA (tRNA), ribosomal RNA (rRNA) etc. of a gene.

15 By "single stranded DNA" (ssDNA) is meant a naturally occurring or synthetic deoxyribonucleic acid molecule comprising a linear single strand, for example, a ssDNA can be a sense or antisense gene sequence or EST (Expressed Sequence Tag).

By "predetermined target" is meant a signaling agent or target signaling agent that is chosen to interact with a nucleic acid sensor molecule to generate a detectable

20 response.

By "validate a predetermined gene target" is meant to confirm that a particular gene is associated with a specific phenotype, disease, or biological function in a system. Once the relationship between a gene and its function or resulting phenotype is determined, the gene can be targeted to modulate the activity of the gene.

25 By "validate a predetermined RNA target" is meant to confirm that a particular RNA transcript of a gene or other RNA is associated with a specific phenotype, disease, or biological function in a system. Once the relationship between the RNA and its

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function or resulting phenotype is determined, the RNA can be targeted to modulate the activity of the RNA or the gene encoding the RNA.

By "validate a predetermined peptide target" is meant to confirm that a particular peptide is associated with a specific phenotype, disease, or biological function in a
5   system. Once the relationship between the peptide and its function or resulting phenotype is determined, the peptide or RNA encoding the peptide can be targeted to modulate the activity of the peptide or the gene encoding the peptide.

By "validate a predetermined protein target" is meant to confirm that a particular protein is associated with a specific phenotype, disease, or biological function in a
10   system. Once the relationship between the protein and its function or resulting phenotype is determined, the protein or RNA encoding the protein can be targeted to modulate the activity of the protein or the gene encoding the protein.

By "SNP" is meant a single nucleotide polymorphism as is known in the art to include single nucleotide substitutions or mismatches in a genome (see Brookes, 1999,
15   *Gene*, 234, 177-186; Stephens, 1999, *Molecular Diagnosis*, 4, 309-317). SNPs can be used to identify genes and gene functions as well as to characterize a genotype.

By "validate a predetermined SNP target" is meant to confirm that a particular SNP of a gene is associated with a specific phenotype, disease, or biological function in a system. Once the relationship between the SNP and its function, associated gene
20 -   function, or resulting phenotype is determined, the SNP can be targeted to modulate the activity of the SNP or the gene associated with the SNP.

By "SNP scoring" is meant a process of identifying and measuring the presence of SNPs in a genome. SNP scoring can also refer to a system of ranking single nucleotide polymorphisms in terms of the relationship between a particular SNP and a certain
25   disease state or drug response in an organism, for example a human. SNP scoring can be used in determining the genotype of an organism.

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By "proteome" is meant the complete set of proteins found in a particular system, such as a cell or organism, for example a human cell or human.

By "proteome map" is meant the functional relationship between different protein constituents of a proteome.

5   By "proteome scoring" is meant a process of identifying and measuring the presence of proteins in a proteome. Proteome scoring can also refer to a system of ranking protiens in terms of the relationship between a particular protein and a certain disease state or drug response in an organism, for example a human. Proteome scoring can be used in determining the phenotype of an organism.

10   By "disease specific proteome" is meant a proteome associated with a particular disease or condition.

By "treatment specific proteome" is meant a proteome associated with a particular treatment or therapy.

By "molecular profiling" is meant the use of nucleic acid sensor molecules for
15   determining the prognosis and monitoring of human disease (e.g., cancer or human infectious disease) outcome in a subject and the use of nucleic acid sensor molecules for monitoring of subjects as a function of an approved drug or a drug under development, and/or subject surveillance and screening for drug and/or drug treatment. Molecular profiling as contemplated by the instant invention can comprise the use of profiling chip
20   technology.

By "profiling chip" is meant a substrate to the surface of which one or more nucleic acid sensor molecules or components thereof are immobilized in a spatially defined and physically addressable manner (also referred to as arrays), for example wherein each nucleic acid sensor molecule immobilized on the substrate is designed to
25   be dependent on a specific target protein co-factor. Each such Target Protein co-factor can be a marker for a specific human disease or condition (e.g., cancer or infectious disease). The profiling chip may be designed for a specific cancer or

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infectious disease or a group of related or unrelated cancers or infectious diseases. The nucleic acid sensor molecules can be immobilized to the surface of the substrate by means of *in situ* synthesis or via linkers. In certain embodiments, the term "profiling chip" shall include an individual chip array or a wafer containing more than one chip array, where the arrays are distinctly separated from each other.

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In certain embodiment, the term "target protein" refers to a protein which can act as the co-factor for nucleic acid sensor molecule activity.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

10   <u>Detection of Target Signaling Molecules</u>

In one embodiment, the invention features several approaches to detecting signaling agents, ligands and/or target signaling molecules in a system using nucleic acid molecules. In all cases, activity of the nucleic acid is modulated via interaction of the nucleic acid with the target signaling agent, ligand and/or target signaling molecule.

15   In one embodiment, the present invention utilizes at least three oligonucleotide sequences for proper function: nucleic acid sensor molecule, reporter molecule, and target signaling molecule. In a non-limiting example, the nucleic acid sensor molecule is comprised of a sensor component and an enzymatic nucleic acid component. The nucleic acid sensor molecule can be further comprised of a linker between the sensor component

20   and the enzymatic nucleic acid component. The nucleic acid sensor molecule is in its inactive state when the sensor component binds to the nucleic acid sensor molecule in the enzymatic nucleic acid component. The sensor component can bind to the substrate binding regions or nucleotides that contribute to the secondary or tertiary structure of the enzymatic nucleic acid component. For example, the sensor component can bind to

25   nucleotides located within the nucleic acid sensor molecule, which can disrupt catalytic activity. The reporter molecule may be able to bind to the nucleic acid sensor molecule, but a catalytic activity would be inhibited since the molecule is structurally inactive. Alternatively, the sensor component can bind to the substrate binding region(s) of the

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enzymatic nucleic acid component, which can prevent the reporter molecule from binding to the nucleic acid sensor molecule. The sensor component cannot be cleaved because the cleavage site would contain either a chemical modification which prevents cleavage or an inappropriate sequence. For example, hammerhead ribozymes need to have a NUH

5   motif in the molecule to be cleaved (H is adenosine, cytidine, or uridine) for proper cleavage. By adding a guanosine at the H position in the RNA to be cleaved, cleavage can be inhibited.

In the presence of the target signaling molecule, the sensor component can disassociate from the enzymatic nucleic acid component and bind to the target signaling

10   molecule preferentially. The sensor component can preferentially bind to the target signaling molecule which results in the formation of a more stable complex. For example, the sensor component can bind to more nucleotides on the target signaling molecule than on the nucleic acid sensor molecule. Binding to a larger number of nucleotides can have increased chemical stability and therefore is preferred over binding

15   to a smaller number of nucleotides.

When the sensor component is bound to the target signaling molecule and the reporter molecule binds to the nucleic acid sensor molecule, a reaction can be catalyzed on the reporter molecule by the enzymatic nucleic acid component. For example, the reporter molecule can be cleaved. The cleavage event can then be detected by using a

20   number of assays. For example, electrophoresis on a polyacrylamide gel would detect not only the full length reporter oligonucleotide but also any cleavage products that were created by the functional nucleic acid sensor molecule. The detection of these cleavage products indicate the presence of the target signaling molecule. In addition, the reporter molecule can contain a fluorescent molecule at one end which fluorescence signal is

25   quenched by another molecule attached at the other end of the reporter molecule. Cleavage of the reporter molecule in this case results in the disassociation of the florescent molecule and the quench molecule, resulting in a signal. This signal can be detected and/or quantified by methods known in the art (for example see Nathan *et al.*, US Patent No. 5,871,914, Birkenmeyer, US Patent No. 5,427,930, and Lizardi *et al.*, US

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Patent No. 5,652,107, George *et al.*, US Patent Nos. 5,834,186 and 5,741,679, and Shih *et al.*, US Patent No. 5,589,332).

Alternatively, the sensor of the signaling molecule can comprise a separate oligonucleotide sequence.

5   Target sites

Targets for useful nucleic acid sensor molecules can be determined as disclosed in Draper *et al.*, WO 93/23569; Sullivan *et al.*, WO 93/23057; Thompson *et al.*, WO 94/02595; Draper *et al.*, WO 95/04818; McSwiggen *et al.*, US Patent No. 5,525,468 and hereby incorporated by reference herein in totality. Rather than repeat the guidance
10   provided in those documents here, below are provided specific examples of such methods, not limiting to those in the art. Nucleic acid sensor molecules to such targets are designed as described in those applications and synthesized to be tested *in vitro* and *in vivo*, as also described. Such nucleic acid sensor molecules can also be optimized and delivered as described therein.

15   Hammerhead, hairpin, Inozyme, Zinzyme, Amberzyme and DNAzyme-based nucleic acid sensor molecules are designed that can bind and are individually analyzed by computer folding (Jaeger *et al.*, 1989 *Proc. Natl. Acad. Sci. USA*, 86, 7706; Denman, 1993, *Biotechniques*, 15, 1090) to assess whether the nucleic acid sensor molecule sequences fold into the appropriate secondary structure. Those nucleic acid sensor
20   molecules with unfavorable intramolecular interactions between the binding arms and the catalytic core are eliminated from consideration. Varying binding arm lengths can be chosen to optimize activity. Generally, at least 5 bases on each arm are able to bind to, or otherwise interact with, the target RNA. Nucleic acid molecules of the differing motifs are designed to anneal to various sites in the mRNA message. The binding arms are
25   complementary to the target site sequences described above.

Hammerhead, DNAzyme, NCH, amberzyme, zinzyme or G-Cleaver-based nucleic acid sensor molecule cleavage sites were identified and were designed to anneal to various sites in the RNA target. The binding arms are complementary to the target site sequences described above. The nucleic acid molecules were chemically synthesized.
30   The method of synthesis used follows the procedure for normal DNA/RNA synthesis as

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described below and in Usman *et al.*, 1987 *J. Am. Chem. Soc.*, 109, 7845; Scaringe *et al.*, 1990 *Nucleic Acids Res.*, 18, 5433; Wincott *et al.*, 1995 *Nucleic Acids Res.* 23, 2677-2684; and Caruthers *et al.*, 1992, *Methods in Enzymology* 211,3-19.

<u>Nucleic acid molecule Synthesis</u>

5   The nucleic acid molecules of the invention, including certain nucleic acid sensor molecules, can be synthesized using the methods described in Usman *et al.*, 1987, *J. Am. Chem. Soc.*, 109, 7845; Scaringe *et al.*, 1990, *Nucleic Acids Res.*, 18, 5433; and Wincott *et al.*, 1995, *Nucleic Acids Res.* 23, 2677-2684 Wincott *et al.*, 1997, *Methods Mol. Bio.*, 74, 59. Such methods make use of common nucleic acid protecting and coupling groups,

10   such as dimethoxytrityl at the 5'-end, and phosphoramidites at the 3'-end. In a non-limiting example, small scale syntheses are conducted on a 394 Applied Biosystems, Inc. synthesizer using a 0.2 μmol scale protocol with a 7.5 min coupling step for alkylsilyl protected nucleotides and a 2.5 min coupling step for 2'-O-methylated nucleotides. **Table I** outlines the amounts and the contact times of the reagents used in the synthesis

15   cycle. Alternatively, syntheses at the 0.2 μmol scale can be done on a 96-well plate synthesizer, such as the PG2100 instrument produced by Protogene (Palo Alto, CA) with minimal modification to the cycle. A 33-fold excess (60 μL of 0.11 M = 6.6 μmol) of 2'-O-methyl phosphoramidite and a 75-fold excess of S-ethyl tetrazole (60 μL of 0.25 M = 15 μmol) can be used in each coupling cycle of 2'-O-methyl residues relative to polymer-

20   bound 5'-hydroxyl. A 66-fold excess (120 μL of 0.11 M = 13.2 μmol) of alkylsilyl (ribo) protected phosphoramidite and a 150-fold excess of S-ethyl tetrazole (120 μL of 0.25 M = 30 μmol) can be used in each coupling cycle of ribo residues relative to polymer-bound 5'-hydroxyl. Average coupling yields on the 394 Applied Biosystems, Inc. synthesizer, determined by colorimetric quantitation of the trityl fractions, are typically 97.5-99%.

25   Other oligonucleotide synthesis reagents for the 394 Applied Biosystems, Inc. synthesizer include; detritylation solution is 3% TCA in methylene chloride (ABI); capping is performed with 16% *N*-methyl imidazole in THF (ABI) and 10% acetic anhydride/10% 2,6-lutidine in THF (ABI); oxidation solution is 16.9 mM I₂, 49 mM pyridine, 9% water in THF (PERSEPTIVE™). Burdick & Jackson Synthesis Grade acetonitrile is used

30   directly from the reagent bottle. S-Ethyltetrazole solution (0.25 M in acetonitrile) is made

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up from the solid obtained from American International Chemical, Inc. Alternately, for the introduction of phosphorothioate linkages, Beaucage reagent (3H-1,2-Benzodithiol-3-one 1,1-dioxide 0.05 M in acetonitrile) is used.

Cleavage from the solid support and deprotection of the oligonucleotide is typically
5   performed using either a two-pot or one-pot protocol. For the two-pot protocol, the polymer-bound trityl-on oligoribonucleotide is transferred to a 4 mL glass screw top vial and suspended in a solution of 40% aq. methylamine (1 mL) at 65 °C for 10 min. After cooling to −20 °C, the supernatant is removed from the polymer support. The support is washed three times with 1.0 mL of EtOH:MeCN:H2O/3:1:1, vortexed and the
10   supernatant is then added to the first supernatant. The combined supernatants, containing the oligoribonucleotide, are dried to a white powder. The base deprotected oligoribonucleotide is resuspended in anhydrous TEA/HF/NMP solution (300 μL of a solution of 1.5 mL N-methylpyrrolidinone, 750 μL TEA and 1 mL TEA•3HF to provide a 1.4 M HF concentration) and heated to 65 °C. After 1.5 h, the oligomer is quenched with
15   1.5 M NH4HCO3.

Alternatively, for the one-pot protocol, the polymer-bound trityl-on oligoribonucleotide is transferred to a 4 mL glass screw top vial and suspended in a solution of 33% ethanolic methylamine/DMSO: 1/1 (0.8 mL) at 65°C for 15 min. The vial is brought to r.t. TEA•3HF (0.1 mL) is added and the vial is heated at 65 °C for 15
20   min. The sample is cooled at −20 °C and then quenched with 1.5 M NH4HCO3. An alternative deprotection cocktail for use in the one pot protocol comprises the use of aqueous methylamine (0.5 ml) at 65°C for 15 min followed by DMSO (0.8 ml) and TEA•3HF (0.3 ml) at 65°C for 15 min. A similar methodology can be employed with 96-well plate synthesis formats by using a Robbins Scientific Flex Chem block, in which the
25   reagents are added for cleavage and deprotection of the oligonucleotide.

For anion exchange desalting of the deprotected oligomer, the TEAB solution is loaded onto a Qiagen 500® anion exchange cartridge (Qiagen Inc.) that is prewashed with 50 mM TEAB (10 mL). After washing the loaded cartridge with 50 mM TEAB (10 mL), the RNA is eluted with 2 M TEAB (10 mL) and dried down to a white powder.

For purification of the trityl-on oligomers, the quenched NH_4HCO_3 solution is loaded onto a C-18 containing cartridge that had been prewashed with acetonitrile followed by 50 mM TEAA. After washing the loaded cartridge with water, the RNA is detritylated with 0.5% TFA for 13 min. The cartridge is then washed again with water,

5   salt exchanged with 1 M NaCl and washed with water again. The oligonucleotide is then eluted with 30% acetonitrile. Alternatively, for oligonucleotides synthesized in a 96-well format, the crude trityl-on oligonucleotide is purified using a 96-well solid phase extraction block packed with C18 material, on a Bahdan Automation workstation.

The average stepwise coupling yields are typically >98% (Wincott et al., 1995

10   *Nucleic Acids Res.* 23, 2677-2684). Those of ordinary skill in the art will recognize that the scale of synthesis can be adapted as larger or smaller than the example described above including but not limited to 96 well format, all that is important is the ratio of chemicals used in the reaction.

To ensure the quality of synthesis of nucleic acid molecules of the invention,

15   quality control measures are utilized for the analysis of nucleic acid material. Capillary Gel Electrophoresis, for example using a Beckman MDQ CGE instrument, can be ulitized for rapid analysis of nucleic acid molecules, by introducing sample on the short end of the capillary. In addition, mass spectrometry, for example using a PE Biosystems Voyager-DE MALDI instrument, in combination with the Bohdan workstation, can be utilized in

20   the analysis of oligonucleotides, including oligonucleotides synthesized in the 96-well format.

The nucleic acids of the invention can also be synthesized in two parts and annealed to reconstruct the nucleic acid sensor molecules (Chowrira and Burke, 1992 *Nucleic Acids Res.;* 20, 2835-2840). The nucleic acids are also synthesized enzymatically using a

25   variety of methods known in the art, for example as described in Havlina, International PCT publication No. WO 9967413, or from DNA templates using bacteriophage T7 RNA polymerase (Milligan and Uhlenbeck, 1989, *Methods Enzymol.* 180, 51). Other methods of enzymatic synthesis of the nucleic acid molecules of the invention are generally described in Kim *et al.,* 1995, *Biotechniques,* 18, 992; Hoffman *et al.,* 1994,

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Biotechniques, 17, 372; Cazenare *et al.,* 1994, *PNAS USA,* 91, 6972; Hyman, US Patent No. 5,436,143; and Karpeisky *et al.,* International PCT publication No. WO 98/28317)

Alternatively, the nucleic acid molecules of the present invention can be synthesized separately and joined together post-synthetically, for example by ligation
5 (Moore *et al.,* 1992, *Science* 256, 9923; Draper *et al.,* International PCT publication No. WO 93/23569; Shabarova *et al.,* 1991, *Nucleic Acids Research* 19, 4247; Bellon *et al.,* 1997, *Nucleosides & Nucleotides,* 16, 951; Bellon *et al.,* 1997, *Bioconjugate Chem.* 8, 204).

The nucleic acid molecules of the present invention are preferably modified to
10 enhance stability by modification with nuclease resistant groups, for example, 2'-amino, 2'-C-allyl, 2'-flouro, 2'-O-methyl, 2'-H (for a review see Usman and Cedergren, 1992, *TIBS* 17, 34; Usman *et al.,* 1994, *Nucleic Acids Symp. Ser.* 31, 163). Nucleic acid sensor molecules are purified by gel electrophoresis using known methods or are purified by high pressure liquid chromatography (HPLC; See Wincott *et al.,* Supra, the totality of
15 which is hereby incorporated herein by reference) and are re-suspended in water.

Optimizing nucleic acid molecule activity

Synthesizing nucleic acid molecules with modifications (base, sugar and/or phosphate) that prevent their degradation by serum ribonucleases can increase their potency (see *e.g.,* Eckstein *et al.,* International Publication No. WO92/07065; Perrault *et.*
20 *al.,* 1990 *Nature* 344, 565; Pieken *et al.,* 1991, *Science* 253, 314; Usman and Cedergren, 1992, *Trends in Biochem. Sci.* 17, 334; Usman *et al.,* International Publication No. WO93/15187; Rossi *et al.,* International Publication No. WO 91/03162; Sproat, US Patent No. 5,334,711; and Burgin *et al., supra;* all of these describe various chemical modifications that can be made to the base, phosphate and/or sugar moieties of the
25 nucleic acid molecules described herein. All these references are incorporated by reference herein. Modifications which enhance their efficacy in cells, and removal of bases from nucleic acid molecules to shorten oligonucleotide synthesis times and reduce chemical requirements are preferably desired.

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There are several examples in the art describing sugar, base and phosphate modifications that can be introduced into nucleic acid molecules with significant enhancement in their nuclease stability and efficacy. For example, oligonucleotides are modified to enhance stability and/or enhance biological activity by modification with

5   nuclease resistant groups, for example, 2'-amino, 2'-C-allyl, 2'-flouro, 2'-O-methyl, 2'-H, nucleotide base modifications (for a review see Usman and Cedergren, 1992, *TIBS*. 17, 34; Usman *et al.*, 1994, *Nucleic Acids Symp. Ser.* 31, 163; Burgin *et al.*, 1996, *Biochemistry* , 35, 14090). Sugar modifications of nucleic acid molecules have been extensively described in the art (see Eckstein *et al.*, *International Publication* PCT No.

10   WO 92/07065; Perrault *et al. Nature*, 1990, *344*, 565-568; Pieken *et al.* *Science*, 1991, *253*, 314-317; Usman and Cedergren, *Trends in Biochem. Sci.* , 1992, *17*, 334-339; Usman *et al. International Publication* PCT No. WO 93/15187; Sproat, US Patent No. 5,334,711 and Beigelman *et al.*, 1995, *J. Biol. Chem.*, 270, 25702; Beigelman *et al.*, International PCT publication No. WO 97/26270; Beigelman *et al.*, US Patent No.

15   5,716,824; Usman *et al.*, US patent No. 5,627,053; Woolf *et al.*, International PCT Publication No. WO 98/13526; Thompson *et al.*, USSN-60/082,404 which was filed on April 20, 1998; Karpeisky *et al.*, 1998, *Tetrahedron Lett.*, 39, 1131; Earnshaw and Gait, 1998, *Biopolymers (Nucleic acid Sciences)*, 48, 39-55; Verma and Eckstein, 1998, *Annu. Rev. Biochem.*, 67, 99-134; and Burlina *et al.*, 1997, *Bioorg. Med. Chem.*, 5, 1999-2010;

20   all of the references are hereby incorporated by reference herein in their totalities). Such publications describe general methods and strategies to determine the location of incorporation of sugar, base and/or phosphate modifications and the like into nucleic acid sensor molecule molecules without inhibiting catalysis. In view of such teachings, similar modifications can be used as described herein to modify the nucleic acid

25   molecules of the instant invention.

While chemical modification of oligonucleotide internucleotide linkages with phosphorothioate, phosphorothioate, and/or 5'-methylphosphonate linkages improves stability, many of these modifications can cause some toxicity. Therefore when designing nucleic acid molecules the amount of these internucleotide linkages should be minimized.

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The reduction in the concentration of these linkages should lower toxicity resulting in increased efficacy and higher specificity of these molecules.

Nucleic acid molecules having chemical modifications which maintain or enhance activity are provided. Such nucleic acid is also generally more resistant to nucleases than
5 unmodified nucleic acid. Thus, in the presence of biological fluids, or in cells, the activity can not be significantly lowered. Clearly, nucleic acid molecules must be resistant to nucleases in order to function as effective diagnostic agents, whether utilized *in vitro* and/or *in vivo*. Improvements in the synthesis of RNA and DNA (Wincott *et al.*, 1995 *Nucleic Acids Res.* 23, 2677; Caruthers *et al.*, 1992, *Methods in Enzymology* 211,3-
10 19; Karpeisky *et al.*, International PCT publication No. WO 98/28317) (incorporated by reference herein) have expanded the ability to modify nucleic acid molecules by introducing nucleotide modifications to enhance their nuclease stability as described above.

In another aspect the nucleic acid molecules comprise a 5' and/or a 3'- cap
15 structure.

In one embodiment, the invention features modified nucleic acid molecules with phosphate backbone modifications comprising one or more phosphorothioate, phosphorodithioate, methylphosphonate, morpholino, amidate carbamate, carboxymethyl, acetamidate, polyamide, sulfonate, sulfonamide, sulfamate, formacetal, thioformacetal,
20 and/or alkylsilyl, substitutions. For a review of oligonucleotide backbone modifications see Hunziker and Leumann, 1995, *Nucleic Acid Analogues: Synthesis and Properties*, in *Modern Synthetic Methods*, VCH, 331-417, and Mesmaeker *et al.*, 1994, *Novel Backbone Replacements for Oligonucleotides*, in *Carbohydrate Modifications in Antisense Research*, ACS, 24-39. These references are hereby incorporated by reference herein.

25 In connection with 2'-modified nucleotides as described for the present invention, by "amino" is meant 2'-NH$_2$ or 2'-O- NH$_2$, which can be modified or unmodified. Such modified groups are described, for example, in Eckstein *et al.*, U.S. Patent 5,672,695 and Karpeisky *et al.*, WO 98/28317, respectively, which are both incorporated by reference herein in their entireties.

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Various modifications to nucleic acid (*e.g.*, nucleic acid sensor molecule) structure can be made to enhance the utility of these molecules. Such modifications enhance shelf-life, half-life *in vitro*, stability, and ease of introduction of such oligonucleotides to the target site, *e.g.*, to enhance penetration of cellular membranes, and confer the ability to recognize and bind to targeted cells.

Administration of Nucleic Acid Molecules

Methods for the delivery of nucleic acid molecules are described in Akhtar *et al.*, 1992, *Trends Cell Bio.*, 2, 139; and Delivery Strategies for Antisense Oligonucleotide Therapeutics, ed. Akhtar, 1995, which are both incorporated herein by reference. Sullivan *et al.*, PCT WO 94/02595, further describes the general methods for delivery of enzymatic RNA molecules. These protocols can be utilized for the delivery of virtually any nucleic acid molecule. Nucleic acid molecules can be administered to cells by a variety of methods known to those familiar to the art, including, but not restricted to, encapsulation in liposomes, by iontophoresis, or by incorporation into other vehicles, such as hydrogels, cyclodextrins, biodegradable nanocapsules, and bioadhesive microspheres. Alternatively, the nucleic acid/vehicle combination is locally delivered by direct injection or by use of an infusion pump. Other routes of delivery include, but are not limited to oral (tablet or pill form) and/or intrathecal delivery (Gold, 1997, *Neuroscience*, 76, 1153-1158). Other approaches include the use of various transport and carrier systems, for example though the use of conjugates and biodegradable polymers. For a comprehensive review on drug delivery strategies including CNS delivery, see Ho *et al.*, 1999, *Curr. Opin. Mol. Ther.*, 1, 336-343 and Jain, Drug Delivery Systems: Technologies and Commercial Opportunities, Decision Resources, 1998 and Groothuis *et al.*, 1997, *J. NeuroVirol.*, 3, 387-400. More detailed descriptions of nucleic acid delivery and administration are provided in Sullivan *et al.*, supra, Draper *et al.*, PCT WO93/23569, Beigelman *et al.*, PCT WO99/05094, and Klimuk *et al.*, PCT WO99/04819, all of which are incorporated by reference herein.

The molecules of the instant invention can be used as pharmaceutical agents. Pharmaceutical agents prevent, inhibit the occurrence, or treat (alleviate a symptom to some extent, preferably all of the symptoms) of a disease state in a subject.

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The negatively charged polynucleotides of the invention can be administered (e.g., RNA, DNA or protein) and introduced into a subject by any standard means, with or without stabilizers, buffers, and the like, to form a pharmaceutical composition. When it is desired to use a liposome delivery mechanism, standard protocols for formation of

5   liposomes can be followed. The compositions of the present invention can also be formulated and used as tablets, capsules or elixirs for oral administration; suppositories for rectal administration; sterile solutions; suspensions for injectable administration; and the other compositions known in the art.

The present invention also includes pharmaceutically acceptable formulations of

10   the compounds described. These formulations include salts of the above compounds, e.g., acid addition salts, for example, salts of hydrochloric, hydrobromic, acetic acid, and benzene sulfonic acid.

A pharmacological composition or formulation refers to a composition or formulation in a form suitable for administration, e.g., systemic administration, into a cell

15   or subject, preferably a human. Suitable forms, in part, depend upon the use or the route of entry, for example oral, transdermal, or by injection. Such forms should not prevent the composition or formulation from reaching a target cell (i.e., a cell to which the negatively charged polymer is desired to be delivered to). For example, pharmacological compositions injected into the blood stream should be soluble. Other factors are known

20   in the art, and include considerations such as toxicity and forms which prevent the composition or formulation from exerting its effect.

By "systemic administration" is meant in vivo systemic absorption or accumulation of drugs in the blood stream followed by distribution throughout the entire body. Administration routes which lead to systemic absorption include, without limitations:

25   intravenous, subcutaneous, intraperitoneal, inhalation, oral, intrapulmonary and intramuscular. Each of these administration routes expose the desired negatively charged polymers, e.g., nucleic acids, to an accessible diseased tissue. The rate of entry of a drug into the circulation has been shown to be a function of molecular weight or size. The use of a liposome or other drug carrier comprising the compounds of the instant invention can

30   potentially localize the drug, for example, in certain tissue types, such as the tissues of the

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reticular endothelial system (RES). A liposome formulation which can facilitate the association of drug with the surface of cells, such as, lymphocytes and macrophages is also useful. This approach can provide enhanced delivery of the drug to target cells by taking advantage of the specificity of macrophage and lymphocyte immune recognition of

5   abnormal cells, such as cancer cells.

By pharmaceutically acceptable formulation is meant, a composition or formulation that allows for the effective distribution of the nucleic acid molecules of the instant invention in the physical location most suitable for their desired activity. Non-limiting examples of agents suitable for formulation with the nucleic acid molecules of

10   the instant invention include: PEG conjugated nucleic acids, phospholipid conjugated nucleic acids, nucleic acids containing lipophilic moieties, phosphorothioates, P-glycoprotein inhibitors (such as Pluronic P85) which can enhance entry of drugs into various tissues, for exaple the CNS (Jolliet-Riant and Tillement, 1999, *Fundam. Clin. Pharmacol.*, 13, 16-26); biodegradable polymers, such as poly (DL-lactide-coglycolide)

15   microspheres for sustained release delivery after implantation (Emerich, DF et al, 1999, *Cell Transplant,* 8, 47-58) Alkermes, Inc. Cambridge, MA; and loaded nanoparticles, such as those made of polybutylcyanoacrylate, which can deliver drugs across the blood brain barrier and can alter neuronal uptake mechanisms (Prog Neuropsychopharmacol Biol Psychiatry, 23, 941-949, 1999). Other non-limiting examples of delivery strategies,

20   including CNS delivery of the nucleic acid molecules of the instant invention include material described in Boado *et al.,* 1998, *J. Pharm. Sci.,* 87, 1308-1315; Tyler *et al.,* 1999, *FEBS Lett.,* 421, 280-284; Pardridge *et al.,* 1995, *PNAS USA.,* 92, 5592-5596; Boado, 1995, *Adv. Drug Delivery Rev.,* 15, 73-107; Aldrian-Herrada *et al.,* 1998, *Nucleic Acids Res.,* 26, 4910-4916; and Tyler *et al.,* 1999, *PNAS USA.,* 96, 7053-7058. All these

25   references are hereby incorporated herein by reference.

The invention also features the use of the composition comprising surface-modified liposomes containing poly (ethylene glycol) lipids (PEG-modified, or long-circulating liposomes or stealth liposomes). Nucleic acid molecules of the invention can also comprise covalently attached PEG molecules of various molecular weights. These

30   formulations offer a method for increasing the accumulation of drugs in target tissues.

This class of drug carriers resists opsonization and elimination by the mononuclear
phagocytic system (MPS or RES), thereby enabling longer blood circulation times and
enhanced tissue exposure for the encapsulated drug (Lasic *et al. Chem. Rev.* 1995, 95,
2601-2627; Ishiwata *et al., Chem. Pharm. Bull.* 1995, 43, 1005-1011). Such liposomes

5   have been shown to accumulate selectively in tumors, presumably by extravasation and
capture in the neovascularized target tissues (Lasic *et al., Science* 1995, 267, 1275-1276;
Oku *et al.,*1995, *Biochim. Biophys. Acta,* 1238, 86-90). The long-circulating liposomes
enhance the pharmacokinetics and pharmacodynamics of DNA and RNA, particularly
compared to conventional cationic liposomes which are known to accumulate in tissues

10   of the MPS (Liu *et al., J. Biol. Chem.* 1995, 42, 24864-24870; Choi *et al.,* International
PCT Publication No. WO 96/10391; Ansell *et al.,* International PCT Publication No. WO
96/10390; Holland *et al.,* International PCT Publication No. WO 96/10392; all of which
are incorporated by reference herein). Long-circulating liposomes are also likely to
protect drugs from nuclease degradation to a greater extent compared to cationic

15   liposomes, based on their ability to avoid accumulation in metabolically aggressive MPS
tissues such as the liver and spleen. All of these references are incorporated by reference
herein.

The present invention also includes compositions prepared for storage or
administration which include a pharmaceutically effective amount of the desired

20   compounds in a pharmaceutically acceptable carrier or diluent. Acceptable carriers or
diluents for therapeutic use are well known in the pharmaceutical art, and are described,
for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co. (A.R.
Gennaro edit. 1985) hereby incorporated by reference herein. For example, preservatives,
stabilizers, dyes and flavoring agents can be provided. These include sodium benzoate,

25   sorbic acid and esters of p-hydroxybenzoic acid. In addition, antioxidants and suspending
agents can be used.

A pharmaceutically effective dose is that dose required to prevent, inhibit the
occurrence, or treat (alleviate a symptom to some extent, preferably all of the symptoms)
of a disease state. The pharmaceutically effective dose depends on the type of disease,

30   the composition used, the route of administration, the type of mammal being treated, the

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physical characteristics of the specific mammal under consideration, concurrent medication, and other factors which those skilled in the medical arts will recognize. Generally, an amount between 0.1 mg/kg and 100 mg/kg body weight/day of active ingredients is administered dependent upon potency of the negatively charged polymer.

5   The nucleic acid molecules of the invention and formulations thereof can be administered orally, topically, parenterally, by inhalation or spray or rectally in dosage unit formulations containing conventional non-toxic pharmaceutically acceptable carriers, adjuvants and vehicles. The term parenteral as used herein includes percutaneous, subcutaneous, intravascular (e.g., intravenous), intramuscular, or intrathecal injection or

10   infusion techniques and the like. In addition, there is provided a pharmaceutical formulation comprising a nucleic acid molecule of the invention and a pharmaceutically acceptable carrier. One or more nucleic acid molecules of the invention can be present in association with one or more non-toxic pharmaceutically acceptable carriers and/or diluents and/or adjuvants, and if desired other active ingredients. The pharmaceutical

15   compositions containing nucleic acid molecules of the invention can be in a form suitable for oral use, for example, as tablets, troches, lozenges, aqueous or oily suspensions, dispersible powders or granules, emulsion, hard or soft capsules, or syrups or elixirs.

Compositions intended for oral use can be prepared according to any method known to the art for the manufacture of pharmaceutical compositions and such

20   compositions can contain one or more such sweetening agents, flavoring agents, coloring agents or preservative agents in order to provide pharmaceutically elegant and palatable preparations. Tablets contain the active ingredient in admixture with non-toxic pharmaceutically acceptable excipients that are suitable for the manufacture of tablets. These excipients can be for example, inert diluents, such as calcium carbonate, sodium

25   carbonate, lactose, calcium phosphate or sodium phosphate; granulating and disintegrating agents, for example, corn starch, or alginic acid; binding agents, for example starch, gelatin or acacia, and lubricating agents, for example magnesium stearate, stearic acid or talc. The tablets can be uncoated or they can be coated by known techniques. In some cases such coatings can be prepared by known techniques to delay

30   disintegration and absorption in the gastrointestinal tract and thereby provide a sustained

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action over a longer period. For example, a time delay material such as glyceryl monosterate or glyceryl distearate can be employed.

Formulations for oral use can also be presented as hard gelatin capsules wherein the active ingredient is mixed with an inert solid diluent, for example, calcium carbonate,

5   calcium phosphate or kaolin, or as soft gelatin capsules wherein the active ingredient is mixed with water or an oil medium, for example peanut oil, liquid paraffin or olive oil.

Aqueous suspensions contain the active materials in admixture with excipients suitable for the manufacture of aqueous suspensions. Such excipients are suspending agents, for example sodium carboxymethylcellulose, methylcellulose, hydropropyl-

10   methylcellulose, sodium alginate, polyvinylpyrrolidone, gum tragacanth and gum acacia; dispersing or wetting agents can be a naturally-occurring phosphatide, for example, lecithin, or condensation products of an alkylene oxide with fatty acids, for example polyoxyethylene stearate, or condensation products of ethylene oxide with long chain aliphatic alcohols, for example heptadecaethyleneoxycetanol, or condensation products of

15   ethylene oxide with partial esters derived from fatty acids and a hexitol such as polyoxyethylene sorbitol monooleate, or condensation products of ethylene oxide with partial esters derived from fatty acids and hexitol anhydrides, for example polyethylene sorbitan monooleate. The aqueous suspensions can also contain one or more preservatives, for example ethyl, or n-propyl p-hydroxybenzoate, one or more coloring

20   agents, one or more flavoring agents, and one or more sweetening agents, such as sucrose or saccharin.

Oily suspensions can be formulated by suspending the active ingredients in a vegetable oil, for example arachis oil, olive oil, sesame oil or coconut oil, or in a mineral oil such as liquid paraffin. The oily suspensions can contain a thickening agent, for

25   example beeswax, hard paraffin or cetyl alcohol. Sweetening agents and flavoring agents can be added to provide palatable oral preparations. These compositions can be preserved by the addition of an anti-oxidant such as ascorbic acid.

Dispersible powders and granules suitable for preparation of an aqueous suspension by the addition of water provide the active ingredient in admixture with a dispersing or

30   wetting agent, suspending agent and one or more preservatives. Suitable dispersing or

wetting agents or suspending agents are exemplified by those already mentioned above. Additional excipients, for example sweetening, flavoring and coloring agents, can also be present.

Pharmaceutical compositions of the invention can also be in the form of oil-in-water emulsions. The oily phase can be a vegetable oil or a mineral oil or mixtures of these. Suitable emulsifying agents can be naturally-occurring gums, for example gum acacia or gum tragacanth, naturally-occurring phosphatides, for example soy bean, lecithin, and esters or partial esters derived from fatty acids and hexitol, anhydrides, for example sorbitan monooleate, and condensation products of the said partial esters with ethylene oxide, for example polyoxyethylene sorbitan monooleate. The emulsions can also contain sweetening and flavoring agents.

Syrups and elixirs can be formulated with sweetening agents, for example glycerol, propylene glycol, sorbitol, glucose or sucrose. Such formulations can also contain a demulcent, a preservative and flavoring and coloring agents. The pharmaceutical compositions can be in the form of a sterile injectable aqueous or oleaginous suspension. This suspension can be formulated according to the known art using those suitable dispersing or wetting agents and suspending agents that have been mentioned above. The sterile injectable preparation can also be a sterile injectable solution or suspension in a non-toxic parentally acceptable diluent or solvent, for example as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that can be employed are water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil can be employed including synthetic mono-or diglycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

The nucleic acid molecules of the invention can also be administered in the form of suppositories, e.g., for rectal administration of the drug. These compositions can be prepared by mixing the drug with a suitable non-irritating excipient that is solid at ordinary temperatures but liquid at the rectal temperature and will therefore melt in the rectum to release the drug. Such materials include cocoa butter and polyethylene glycols.

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Nucleic acid molecules of the invention can be administered parenterally in a sterile medium. The drug, depending on the vehicle and concentration used, can either be suspended or dissolved in the vehicle. Advantageously, adjuvants such as local anesthetics, preservatives and buffering agents can be dissolved in the vehicle.

5   Dosage levels of the order of from about 0.1 mg to about 140 mg per kilogram of body weight per day are useful in the treatment of the above-indicated conditions (about 0.5 mg to about 7 g per subject per day). The amount of active ingredient that can be combined with the carrier materials to produce a single dosage form varies depending upon the host treated and the particular mode of administration. Dosage unit forms

10   generally contain between from about 1 mg to about 500 mg of an active ingredient.

It is understood that the specific dose level for any particular subject depends upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, sex, diet, time of administration, route of administration, and rate of excretion, drug combination and the severity of the particular disease undergoing

15   therapy.

For administration to non-human animals, the composition can also be added to the animal feed or drinking water. It can be convenient to formulate the animal feed and drinking water compositions so that the animal takes in a therapeutically appropriate quantity of the composition along with its diet. It can also be convenient to present the

20   composition as a premix for addition to the feed or drinking water.

The nucleic acid molecules of the present invention can also be administered to a subject in combination with other therapeutic compounds to increase the overall therapeutic effect. The use of multiple compounds to treat an indication can increase the beneficial effects while reducing the presence of side effects.

25   Alternatively, certain of the nucleic acid molecules of the instant invention can be expressed within cells from eukaryotic promoters (e.g., Izant and Weintraub, 1985, Science, 229, 345; McGarry and Lindquist, 1986, Proc. Natl. Acad. Sci., USA 83, 399; Scanlon et al., 1991, Proc. Natl. Acad. Sci. USA, 88, 10591-5; Kashani-Sabet et al., 1992, Antisense Res. Dev., 2, 3-15; Dropulic et al., 1992, J. Virol., 66, 1432-41;

30   Weerasinghe et al., 1991, J. Virol., 65, 5531-4; Ojwang et al., 1992, Proc. Natl. Acad.

Sci. USA, 89, 10802-6; Chen *et al.*, 1992, *Nucleic Acids Res.*, 20, 4581-9; Sarver *et al.*, 1990 *Science*, 247, 1222-1225; Thompson *et al.*, 1995, *Nucleic Acids Res.*, 23, 2259; Good *et al.*, 1997, *Gene Therapy*, 4, 45; Skillern *et al.*, International PCT Publication No. WO 00/22113; Conrad, International PCT Publication No. WO 00/22114; and Conrad,

5   US 6,054,299; all of these references are hereby incorporated in their totalities by reference herein). Those skilled in the art realize that any nucleic acid can be expressed in eukaryotic cells from the appropriate DNA/RNA vector. The activity of such nucleic acids can be augmented by their release from the primary transcript by a enzymatic nucleic acid (Draper *et al.*, PCT WO 93/23569, and Sullivan *et al.*, PCT WO 94/02595;

10   Ohkawa *et al.*, 1992, *Nucleic Acids Symp. Ser.*, 27, 15-6; Taira *et al.*, 1991, *Nucleic Acids Res.*, 19, 5125-30; Ventura *et al.*, 1993, *Nucleic Acids Res.*, 21, 3249-55; Chowrira *et al.*, 1994, *J. Biol. Chem.*, 269, 25856; all of these references are hereby incorporated in their totalities by reference herein). Gene therapy approaches specific to the CNS are described by Blesch *et al.*, 2000, *Drug News Perspect.*, 13, 269-280; Peterson *et al.*,

15   2000, *Cent. Nerv. Syst. Dis.*, 485-508; Peel and Klein, 2000, *J. Neurosci. Methods*, 98, 95-104; Hagihara *et al.*, 2000, *Gene Ther.*, 7, 759-763; and Herrlinger *et al.*, 2000, *Methods Mol. Med.*, 35, 287-312. AAV-mediated delivery of nucleic acid to cells of the nervous system is further described by Kaplitt *et al.*, US 6,180,613.

In another aspect of the invention, nucleic acid molecules of the present invention

20   are preferably expressed from transcription units (see for example Couture *et al.*, 1996, *TIG.*, 12, 510, Skillern *et al.*, International PCT Publication No. WO 00/22113, Conrad, International PCT Publication No. WO 00/22114, and Conrad, US 6,054,299) inserted into DNA or RNA vectors. The recombinant vectors are preferably DNA plasmids or viral vectors. Ribozyme expressing viral vectors can be constructed based on, but not

25   limited to, adeno-associated virus, retrovirus, adenovirus, or alphavirus. Preferably, the recombinant vectors capable of expressing the nucleic acid molecules are delivered as described above, and persist in target cells. Alternatively, viral vectors can be used that provide for transient expression of nucleic acid molecules. Such vectors can be repeatedly administered as necessary. Once expressed, the nucleic acid molecule binds to

30   the target mRNA. Delivery of nucleic acid molecule expressing vectors can be systemic,

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such as by intravenous or intra-muscular administration, by administration to target cells ex-planted from the subject followed by reintroduction into the subject, or by any other means that would allow for introduction into the desired target cell (for a review see Couture *et al.*, 1996, *TIG.*, 12, 510).

5   In one aspect the invention features an expression vector comprising a nucleic acid sequence encoding at least one of the nucleic acid molecules of the instant invention is disclosed. The nucleic acid sequence encoding the nucleic acid molecule of the instant invention is operable linked in a manner which allows expression of that nucleic acid molecule.

10   In another aspect the invention features an expression vector comprising: a) a transcription initiation region (*e.g.*, eukaryotic pol I, II or III initiation region); b) a transcription termination region (*e.g.*, eukaryotic pol I, II or III termination region); c) a nucleic acid sequence encoding at least one of the nucleic acid catalyst of the instant invention; and wherein said sequence is operably linked to said initiation region and said

15 termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule. The vector can optionally include an open reading frame (ORF) for a protein operably linked on the 5' side or the 3'-side of the sequence encoding the nucleic acid catalyst of the invention; and/or an intron (intervening sequences).

Transcription of the nucleic acid molecule sequences are driven from a promoter

20 for eukaryotic RNA polymerase I (pol I), RNA polymerase II (pol II), or RNA polymerase III (pol III). Transcripts from pol II or pol III promoters are expressed at high levels in all cells; the levels of a given pol II promoter in a given cell type depends on the nature of the gene regulatory sequences (enhancers, silencers, etc.) present nearby. Prokaryotic RNA polymerase promoters are also used, providing that the prokaryotic

25 RNA polymerase enzyme is expressed in the appropriate cells (Elroy-Stein and Moss, 1990, *Proc. Natl. Acad. Sci. U S A*, 87, 6743-7; Gao and Huang 1993, *Nucleic Acids Res..*, 21, 2867-72; Lieber *et al.*, 1993, *Methods Enzymol.*, 217, 47-66; Zhou *et al.*, 1990, *Mol. Cell. Biol.*, 10, 4529-37). All of these references are incorporated by reference herein. Several investigators have demonstrated that nucleic acid molecules, such as

30 ribozymes expressed from such promoters can function in mammalian cells (e.g.

Kashani-Sabet *et al.*, 1992, *Antisense Res. Dev.*, 2, 3-15; Ojwang *et al.*, 1992, *Proc. Natl. Acad. Sci. U S A*, 89, 10802-6; Chen *et al.*, 1992, *Nucleic Acids Res.*, 20, 4581-9; Yu *et al.*, 1993, *Proc. Natl. Acad. Sci. U S A*, 90, 6340-4; L'Huillier *et al.*, 1992, *EMBO J.*, 11, 4411-8; Lisziewicz *et al.*, 1993, *Proc. Natl. Acad. Sci. U. S. A*, 90, 8000-4;

5   Thompson *et al.*, 1995, *Nucleic Acids Res.*, 23, 2259; Sullenger & Cech, 1993, *Science*, 262, 1566; all of these references are incorporated by reference herein). More specifically, transcription units such as the ones derived from genes encoding U6 small nuclear (snRNA), transfer RNA (tRNA) and adenovirus VA RNA are useful in generating high concentrations of desired RNA molecules such as ribozymes in cells

10   (Thompson *et al.*, *supra*; Couture and Stinchcomb, *1996, supra*; Noonberg *et al.*, 1994, *Nucleic Acid Res.*, 22, 2830; Noonberg *et al.*, US Patent No. 5,624,803; Good *et al.*, 1997, *Gene Ther.*, 4, 45; Beigelman *et al.*, International PCT Publication No. *WO 96/18736*; all of these publications are incorporated by reference herein. The above ribozyme transcription units can be incorporated into a variety of vectors for introduction

15   into mammalian cells, including but not restricted to, plasmid DNA vectors, viral DNA vectors (such as adenovirus or adeno-associated virus vectors), or viral RNA vectors (such as retroviral or alphavirus vectors) (for a review see Couture and Stinchcomb, *1996, supra*).

In another aspect the invention features an expression vector comprising nucleic

20   acid sequence encoding at least one of the nucleic acid molecules of the invention, in a manner which allows expression of that nucleic acid molecule. The expression vector comprises in one embodiment; a) a transcription initiation region; b) a transcription termination region; c) a nucleic acid sequence encoding at least one said nucleic acid molecule; and wherein said sequence is operably linked to said initiation region and said

25   termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule.

In another embodiment the expression vector comprises: a) a transcription initiation region; b) a transcription termination region; c) an open reading frame; d) a nucleic acid sequence encoding at least one said nucleic acid molecule, wherein said sequence is

30   operably linked to the 3'-end of said open reading frame; and wherein said sequence is

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operably linked to said initiation region, said open reading frame and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule. In yet another embodiment the expression vector comprises: a) a transcription initiation region; b) a transcription termination region; c) an intron; d) a nucleic acid

5   sequence encoding at least one said nucleic acid molecule; and wherein said sequence is operably linked to said initiation region, said intron and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule.

In another embodiment, the expression vector comprises: a) a transcription initiation region; b) a transcription termination region; c) an intron; d) an open reading

10   frame; e) a nucleic acid sequence encoding at least one said nucleic acid molecule, wherein said sequence is operably linked to the 3'-end of said open reading frame; and wherein said sequence is operably linked to said initiation region, said intron, said open reading frame and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule.

15   <u>Examples</u>

The following are non-limiting examples showing techniques useful in isolating nucleic acid molecules of the instant invention.

<u>Example 1: Half-zinzyme nucleic acid sensor molecule (Halfzyme)</u>

Applicant has developed a generalizable methodology for the production of nucleic

20   acid sensor molecules that are activated by target nucleic acids. This technology is based on enzymatic nucleic acids that, in the absence of a target nucleic acid, are catalytically inactive because they lack portions of the catalytic core and substrate recognition elements. In this 'half-ribozyme' or 'halfzyme' system, catalysis can occur if a specific target nucleic acid supplies the sequences required for catalysis in *trans*.

25   Although many enzymatic nucleic acid motifs can be used for the halfzyme strategy, one system uses the Zinzyme motif (Figure 3) in which the substrate nucleic acid is attached to the enzymatic nucleic acid. This motif is small (about 32 nucleotides), carries modifications that confer a half-life in serum of greater than 100 hours, and has minimal target sequence requirements (5'-N3-RG-N3-3', where N= any nucleotide and

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R= A or G). Thus, this motif is readily synthesized, has the ability to detect different sequences, and can be used directly in serum or other biological fluids.

Applicant has tested the feasibility of the halfzyme approach using the Zinzyme motif and the Hepatitis C Virus genome as a model target. A synthetic oligoribonucleotide representing loop IIIB of the 5' untranslated region (UTR), a universally conserved region of the HCV genome, activates catalysis of a rationally designed, sequence matched halfzyme. In the absence of oligoribonucleotide target no nucleic acid sensor molecule activity is detected. Other regions of the HCV 5'-UTR (see Figure 6) can be similarly used in the design of other halfzymes contemplated by the invention.

In this example, the halfzyme is activated by a target sequence derived from intact HCV genome. The 5'-UTR of HCV folds into a compact three-dimensional structure independent of the remaining portion of the HCV genome. To disrupt this structure so that UTR-derived loop IIIB sequences are accessible for activation of the halfzyme, a simple 20 minute pre-treatment step was inserted into the assay. Pre-treatment of the HCV 5'-UTR with a DNA oligonucleotide complementary to stem III and RNase H (Figure 2a) is sufficient to activate halfzyme catalysis to the same extent as that observed with a short synthetic oligoribonucleotide (Figure 2b). Thus, the halfzyme used in these studies can efficiently detect the presence of a conserved sequence element derived from the HCV genome. Target capture by a halfzyme is determined by the affinity of the halfzyme for its target and can be described in molarity by a dissociation constant. The value of this dissociation constant can be rationally engineered into the halfzyme, allowing 100% target capture when halfzyme used in the assay is in excess of this concentration.

A primary concern of any technology aimed at detecting low concentrations of nucleic acids is its sensitivity. The halfzyme approach is unique because catalysis is only promoted in the presence of a sequence-matched target and because 100% target capture can be achieved by manipulating halfzyme concentration. Therefore, single molecule detection is theoretically possible by this approach provided that an adequate signal

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amplification system is in place. Given the enormous flexibility of possible signal amplification and detection systems accommodated by the technology, signal detection should not define the limit of sensitivity of this technology. In practice, the limit of sensitivity of this approach is dictated by the uncatalyzed rate of substrate cleavage promoted under the assay conditions used. Therefore, the salient issue in terms of sensitivity becomes the relative rate of catalyzed versus uncatalyzed substrate cleavage. A virtue of the system is that both the assay conditions and halfzyme activity can be manipulated to maximize this rate differential.

Figure 1 shows a non-limiting example of a "half-zinzyme" nucleic acid sensor molecule with a PEG linker that is modulated by the 5'-UTR of the Hepatitis C virus (HCV 5'-UTR). The figure shows both inactive and active forms of the zinzyme sensor molecule (SEQ ID NO. 43). In the presence of the target signaling oligonucleotide (SEQ ID NO. 26) which represents the stem loop IIIB of the HCV 5'-UTR, the zinzyme sensor demonstrates an activity increase of three logs in cleaving the reporter molecule component of the sensor molecule as shown in the graph (+ oligo target) as compared to the sensor molecule in the absence of the target. In the presence of the full length 350 nt. HCV 5'-UTR, the zinzyme sensor molecule demonstrates an almost one log increase in activity in cleaving the reporter molecule component of the sensor molecule. Reaction conditions: 140mM KCl, 10mM NaCl, 20 mM HEPES pH 7.4, 1mM MgCl2, 1mM CaCl2, 400 nM Nucleic acid sensor, 400 nM Target, Trace of labeled reporter (~10 nM), 25μl reaction volume, Nucleic acid sensor, target and reporter were heated at 75°C for 3 min, cooled to 37°C and cleavage initiated by the addition of MgCl2 and CaCl2.

Example 2: Nucleic Acid Sensor Ligase

A ligase derived from the Bartel class I ligase (Ekland et al., 1995, Science, 269, 364-370) was prepared. Three different constructs carried various 3' truncations. These segments were supplied in trans as oligonulceotide HCV sequence. One ligase, termed HZBART-2 showed ligation rate 107 fold above background ligation (Figure 5).

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Ligation reactions were performed at room temperature in 30 mM Tris, pH 7.5, 200 mM KCl, 60 mM MgCl2 and 0.6 mM EDTA. Halfzyme ligases (1 μM) with corresponding effector oligonulceotide (1 μM) were heated in water at 90°C for 2´min and cooled at room temperature for 10 min followed by the addition of salt, buffer and 32P-labeled substrate oligonucleotide (0.1 mM final concentration). Reactions were carried out for 60 min at room temperature and stopped by the addition of 1 volume of gel loading buffer (7M urea, 100 mM EDTA) and snap cooling on ice. Products were separated on 20% denaturing polyacrylamide gel electrophoresis.

Example 11: Halfzyme SNP discrimination

A halfzyme, based on a zinzyme enzymatic nucleic acid motif, (AZB7.1) was used to discriminate single nucleotide polymorphisms in a nucleic acid sequence derived from HBV (for example GenBank Accession No. AF100308.1). The design of the halfzyme and the sequences used for detecting single nucleotide substitutions within a target sequence are shown in Figure 7. The cognate HBV DNA sequence used contains the sequence 3'-TCGCGGCTGCCC-5' (SEQ ID NO: 51). Two deoxy-guanosine nucleotides within the cognate sequence were each systematically replaced with alternate deoxy nucleotides (c, t, or a) and cleavage activity of the halfzyme (SEQ ID NO: 50) assessed for each single nucleotide substitution in the target sequence. As shown in Figure 8, efficient halfzyme cleavage takes place in the presence of the cognate DNA sequence (SEQ ID NO: 51) and a corresponding all RNA sequence (HBV 1433, SEQ ID NO: 58). However, the introduction of single nucleotide changes within the target sequence (SEQ ID NOS: 52-57) results in loss of cleavage activity at both positions tested within the sequence. This study demonstrates that nucleic acid sensor molecules of the invention, specifically halfzymes, can be used to detect single nucleotide polymorphisms in a target nucleic acid sequence.

Each reaction of the study contained a certain amount of DNA target to be analyzed, 10uM of 32P labeled halfzyme AZB7.1 in 10ul of 1X Buffer (20mM MES pH6.0, 14mM KCl and 10mM NaCl) with 10ng/ul Monkey Genomic DNA and 1mM

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CaCl$_2$ and 1mM MgCl$_2$. Reactions were assembled with all components except the CaCl$_2$ and MgCl$_2$, heated to 80°C for 5 mins, then cooled to 32°C slowly. The reactions were initiated with the addition of the CaCl$_2$ and MgCl$_2$, and incubated overnight. The reactions were terminated by the addition of 10ul of XC/BPB loading dye. The products

5 were resolved by electrophoresis through a 15% denaturing polyacrylamide gel (19:1 cross link) with 7M urea in 1X TBE buffer. The gel was visualized by phosphoimager analysis.

Example 12: Monitoring post-translational modification of proteins in solution with nucleic acid sensor molecules

10 A pre-existing RNA ligand specific for the unphosphorylated form of ERK2 was linked to a variant of the hammerhead ribozyme through a destabilized stem II structure (ERK-HH, **Figure 9A**). Biochemical and structural studies have demonstrated that activity of the hammerhead ribozyme motif requires formation of stem II. Consequently, a reasonable strategy is to induce formation of stem II through molecule binding to an

15 appended RNA ligand. Protein binding can serve to induce ribozyme activity by stabilizing stem II since association of ERK2 with the RNA ligand requires at least partial formation of stem II in the fusion construct. To further disfavor stem II formation in the absence of ERK2, a substrate RNA binding arm in ERK-HH was made complementary to sequences in the destabilized stem II structure in order to form an alternate ERK-HH

20 conformer incapable of cleaving substrate RNA (boxed regions, **Figure 9A**). Upon ERK2 association, this alternate pairing arrangement should be prohibited and substrate RNA, such as a reporter molecule, can therefore associate with, and consequently be cleaved by, ERK-HH.

Nucleic acid sensor molecule activity assays were performed in the presence or

25 absence of ERK2 to assess protein-dependent nucleic acid sensor molecule activation. Cleavage reactions contained 10 mM Tris-HCl, pH 7.5, 10 mM MgCl$_2$, 0.05 µg/µl tRNA, 100 nM nucleic acid sensor molecule, 500 nM protein (or the concentration indicated), and trace 5'-P^{32}-labeled substrate RNA. Recombinant rat ERK2 was produced and

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purified as described in Golden *et al.*, 2000, *J. Biotechnol.*, 81, 167. In all reactions, the level of substrate RNA cleavage in the absence of nucleic acid sensor molecule was subtracted from the level of substrate RNA cleavage in the presence of nucleic acid sensor molecule. Data represent the average values from two or more experiments. ERK-HH displayed little activity in the absence of the ERK2 protein (k_{obs}= 4.2 X 10^{-5} min^{-1}) (Figure 9B). However, unphosphorylated ERK2 stimulated the observed rate by approximately 50 fold ($k_{obs \, +ERK2}$ = 2.1 X 10^{-3} min^{-1}). The observed rate of substrate RNA cleavage by ERK-HH in the presence of ERK2 did not display a log–linear relationship with pH but rather was independent of pH (Figure 9C), suggesting that a conformational rearrangement of the enzymatic nucleic acid domain is the rate-limiting step in product formation. Importantly, catalysis promoted by a nucleic acid sensor molecule containing a mutated RNA ligand that does not associate with ERK2 was unaffected by the presence of ERK2, and was equivalent to the activity of ERK-HH in the absence of ERK2 (ERK-HH/M1, Figure 9B). Thus, the catalytic activation of ERK-HH in the presence of ERK2 results from its capacity to recognize ERK2.

The rational design strategy used to create ERK-HH differs from previous allosteric ribozyme design strategies in that it employs ERK2-modulated sequestration of a substrate RNA binding element (boxed region, Figure 9A). To determine the importance of this novel design element, a version of ERK-HH was constructed such that the sequences in stem I are unable to interact with stem II sequences (ERK-HH/M2; inset in Figure 9D). The appropriate substrate (reporter molecule) for this nucleic acid sensor molecule was cleaved at nearly the same rate and to nearly the same extent in the presence or absence of ERK2 (Figure 9D), suggesting that this design element plays a dominant role in protein-mediated activation of ERK-HH. Interestingly, the observed rate of substrate RNA cleavage promoted by ERK-HH/M2 was approximately twenty-fold greater than the ERK2-stimulated rate of ERK-HH. Thus, the rate-limiting conformational rearrangement of ERK-HH evidenced by the pH independence of substrate RNA cleavage (Figure 9C) may involve the alternate pairing of stem regions I and II. The production of nucleic acid sensor molecules with an even greater rate

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induction by ERK2 may be accomplished by further engineering of ERK-HH to tune the protein dependence of this conformational rearrangement.

Importantly, ERK-HH activity was responsive to the concentration of ERK2 (Figure 10). Maximal activation occurred in the presence of 500 nM ERK2, and 5   activation was observed with as little as 5 nM ERK2 (Figure 10). The ability of ERK-HH activity to monitor low nanomolar concentrations of ERK2 sets this nucleic acid sensor molecule apart from previously reported allosteric ribozymes, which respond to micromolar through millimolar concentrations of their cognate targets. This enhanced sensitivity reflects the use of an RNA ligand domain (sensor domain) in ERK-HH that 10   displays nanomolar affinity for ERK2. Detection of even lower levels of protein target is possible through 'affinity maturation', a technique that has been used to increase the sensitivity of small molecule detection by allosteric ribozymes by over one hundred fold (Soukup et al., 2001, RNA, 7, 524). Alternatively, increased sensitivity of detection is possible by further increasing the rate differential between ERK2-stimulated and ERK2- 15   indepdendent ERK-HH catalysis. Given that the proven detection limit of RNA reagents is equivalent to antibodies (Golden et al., 2000, J. Biotechnol., 81, 167), protein-activated nucleic acid sensor molecules therefore should ultimately prove to be useful alternatives to antibodies in certain applications.

Since nucleic acid ligands developed through combinatorial methods can 20   discriminate between protein isoforms and activation states, the specificity of protein-dependent ERK-HH activation was examined. As expected, bovine serum albumin (BSA) failed to activate ERK-HH above the level seen in the absence of any protein (Figure 11A). More importantly, p38α and JNK2, MAPKs that are 45 % similar to ERK2, failed to stimulate ERK-HH activity (Figure 3, 39A), demonstrating selectivity of this nucleic 25   acid sensor molecule . Because RNA ligands can recognize conformational epitopes and because phosphorylation of ERK2 leads to kinase activation by promoting a conformational change, applicant examined whether ERK-HH was selectively activated by a specific phosphorylation state of ERK2. In contrast to unphosphorylated ERK2, phosphorylated ERK2 (T_{183}, Y_{185}-doubly phosphorylated ERK2; ppERK2) afforded

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minimal nucleic acid sensor molecule activation as judged by the low plateau level of cleavage in the presence of a molar excess of ppERK2 (Figure 11B). Such phosphorylation-state specificity indicates that ERK-HH activation through a nonspecific RNA chaperone effect is unlikely. Analysis of ppERK2 by polyacrylamide gel electrophoresis demonstrates that approximately 10 % of the ppERK2 preparation comprises unphosphorylated protein (inset, Figure 11B); a percentage that correlated well with the relative plateau level of cleavage observed with ppERK2 (8.1 %). Therefore, the low level of ERK-HH activity seen with the preparation of phosphorylated ERK2 most likely reports the small amount of contaminating unphosphorylated protein present in the ppERK2 preparation. Consequently, this protein-activated nucleic acid sensor molecule not only differentiates between ERK2 and MAPKs involved in other cellular processes, it also successfully monitors the post-translational activation state of ERK2.

To serve as useful protein detection reagents, protein-activated nucleic acid sensor molecules should be able to detect their targets in complex mixtures of proteins. To examine this, ERK-HH was tested for its ability to monitor ERK2 in mammalian cell lysates. Exogenous ERK2 was added to aliquots of lysate to levels between 1 % (500 nM) and 0.1 % (50 nM) of the total protein by weight (Figure 12A), concentrations of recombinant ERK2 that can be detected if purified (Figure 10). ERK-HH faithfully reported the concentration of exogenous ERK2 in these samples with an activity that was reduced only two fold relative to its activation with purified ERK2 (Figure 12B). Thus, these data demonstrate that a protein-activated nucleic acid sensor molecule can quantitatively detect its target in a complex mixture of cellular proteins and other macromolecules with only a slightly reduced capacity.

Because allosteric ribozymes couple analyte recognition and signaling in a single molecular event, we examined whether a protein-activated nucleic acid sensor molecule could monitor its target in a solution phase assay. To investigate whether a Fluorescence Resonance Energy Transfer (FRET)-based method could detect the activity of ERK-HH, an assay was developed in which ERK-HH separated a fluorescein dye from a fluorescein

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dye quencher that were coupled to opposite ends of a substrate RNA. The reactions were performed in 450 µl assays containing 100 nM substrate RNA for ERK-HH (5'-fluorescein-ggaacgUCGucacgc-BHQ-3', SEQ ID NO: 59) and 100 nM substrate RNA for a constitutive ribozyme (5'-Cy3-ugagcUGcacugc-BHQ-3', SEQ ID NO: 60) obtained from

5   Integrated DNA Technologies, U.S.A. (lower case = 2'-O-methyl ribonucleotide, BHQ = Black Hole Quencher™). Reaction conditions were identical to standard conditions described previously, except that sodium and potassium salts at final concentrations of 10 mM and 14 mM, respectively, were included; a requirement for activity of the constitutive ribozyme motif. Emission at 517 nm and 568 nm was measured during the

10   initial rate phase of reactions (5.5 hours). A constitutive ribozyme that cleaved a substrate RNA carrying a similarly quenched cyanine 3 (Cy3) fluorophore was used as a normalization control in the reactions (Figure 13A). Emission at 517 nm due to catalysis by ERK-HH increased as the ERK2 concentration increased (Figure 13B), while the activity of the constitutive ribozyme was unaffected by the presence of ERK2 as judged

15   by emission at 568 nm (signal varied less than 3.2 % in all measurements). The ratio of fluorescein emission to Cy3 emission provides a normalized index of ERK-HH activation (right ordinate, Figure 13B); this profile correlated well with that observed in reactions employing radiolabeled substrate RNA and gel electrophoresis to detect ERK-HH activation (Figure 10). These results show that nucleic acid sensor molecules can be used

20   to quantitatively detect a target protein in a simple solution phase assay.

To test the generality of the design principles used to construct ERK-HH, a second protein-activated nucleic acid sensor molecule was constructed (ppERK-HH, Figure 14A). In ppERK-HH, the high affinity ligand specific for unphosphorylated ERK2 was replaced with a high affinity RNA ligand specific for phosphorylated and activated ERK2

25   (Seiwert et al., 2001, Chem. Biol., 7, 833). Otherwise, ERK-HH and ppERK-HH are identical. The rate of substrate RNA (reporter molecule) cleavage promoted by ppERK-HH in the absence of protein was comparable to the uncatalyzed rate of phosphodiester bond hydrolysis of RNA under similar conditions (5.2×10^{-7} min^{-1} at pH 7.5 versus 1.9×10^{-6} min^{-1} at pH 8.0, respectively). However, phosphorylated ERK2 stimulated the

30   observed rate of cleavage by ppERK-HH by ~230-fold (Figure 14B). Importantly,

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unphosphorylated ERK2 failed to activate catalysis by ppERK-HH to a level any greater than that observed in the absence of protein (Figure 14B). Phosphorylated forms of related MAPKs (e.g., p38α and JNK2) which do not bind to the RNA ligand in ppERK-HH also failed to activate catalysis by ppERK-HH. Thus, although further combinatorial

5   selection or rational engineering of protein-activated nucleic acid sensor molecules may be required to enhance catalytic rates, the rational design principles introduced here were generally applicable to develop nucleic acid sensor molecules capable of monitoring protein post-translational modifications.

Allosteric ribozymes have been described that respond to a variety of compounds.
10   Here, applicant demonstrates that nucleic acid sensor molecules have sufficient specificity to also monitor the phosphorylation state of a target protein. The particular example involving selective activation of ERK-HH and ppERK-HH by opposite phosphorylation states of ERK2 (Figures 39B and 42B) is noteworthy because high resolution structural studies indicate that fewer than 10 % of the amino acids in ERK2
15   differ in relative position by more than 1.1Å upon phosphorylation (Canagarajah *et al.*, 1997, *Cell*, 90, 859). Such specificity is ultimately a manifestation of the robustness of RNA combinatorial procedures which, in contrast to the specificity displayed by antibodies, can be readily defined and controlled.

The mechanism of activation of ERK-HH and ppERK-HH by their target analytes
20   differs from that proposed for previously reported allosteric ribozymes: namely, it relies on an alternate conformer to diminish nucleic acid sensor molecule activity in the absence of target protein (Figure 9A and 37D). The strategy introduced here represents a general method for the production of protein-activated nucleic acid sensor molecules (Figures 37 and 42). Since this approach involves the generation of an inactive conformer by the
25   sequestration of a substrate nucleic acid binding element, it should be equally applicable to enzymatic nucleic acid ligases and to enzymatic nucleic acids that carry modifications that confer stability in biological fluids.

A unique advantage of nucleic acid sensor molecules as protein sensing reagents is that they directly couple molecular recognition to signal generation and therefore provide

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simple assays for quantitative protein detection. A nucleic acid sensor molecule assay can simply involve adding nucleic acid sensor molecule and reporter substrate to a solution containing the molecular target, incubating the mixture, and measuring the nucleic acid sensor molecule activity (Figure 13). The ability of nucleic acid sensor molecules to

5   function in parallel in complex mixtures (Figure 12) indicates the feasibility of using several nucleic acid sensor molecules to simultaneously monitor multiple classes of protein analytes in solution (Figure 13). Nucleic acid sensor molecules also function well on solid supports that are suitable for more global profiling of protein expression using high density arrays. Taken together with the ability to produce large numbers of different

10   functional RNAs through automated combinatorial selection, protein-responsive nucleic acid sensor molecules therefore can represent valuable reagents to globally monitor post-translational modifications of proteins in an arrayed format. Such flexibility in assay formats forecasts valuable roles for protein-activated nucleic acid sensor molecules in biological research and molecular diagnostics.

15   <u>Example 13: Selection and Characterization of HCV-Halfzyme nucleic acid sensor molecule</u>

This example summarizes the results of a study investigating the development and use of Halfzyme™ Technology for the sensitive detection of nucleic acids. Halfzymes in this example are a class of RNA-based enzymes that, in the presence of a nucleic acid

20   targeted for detection, direct the ligation of two reporter substrate oligonucleotides through a catalytic reaction. These Halfzyme enzymatic nucleic acids can be multiple turnover enzymes such that a single targeted nucleic acid-activated Halfzyme enzymatic nucleic acid produces many ligation products. Thus, Halfzyme enzymatic nucleic acid Technology provides an amplification step for nucleic acid detection.

25   The study was directed at the detection of sequences present in the Hepatitis C Virus (HCV) genome. Sequences in the 5' untranslated region (UTR) were chosen as the target nucleic acid sequence because of their high degree of sequence conservation among different HCV strains.

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Activities performed in the course of this study included: 1) development of efficient Halfzymes activated by HCV sequences (HCV-Halfzymes) through a robust, *in vitro* combinatorial process referred to as Directed Molecular Evolution (DME), 2) biochemical characterization and optimization of HCV-Halfzymes that function as

5   multiple turnover enzymes, 3) determination of the limit of detection (L.O.D.) afforded by HCV-Halfzymes in an unformatted assay.

Applicant performed L.O.D. determinations in an unformatted assay in which HCV-Halfzyme ligation products were directly visualized and quantified. The L.O.D. assay utilized partially labeled substrate (therefore, less than 1.5% of products could be

10   detected) and an instrument to quantify ligation products

The L.O.D. of HCV sequence detection in this unformatted assay was 6000 molecules when a positive signal was judged as two standard deviations above noise (2 SD L.O.D.). This is a very conservative estimate of the unformatted assay L.O.D. since some experiments yielded detection of a little as 60 molecules and 2 SD L.O.D. values as

15   low as 600 molecules. The kinetic characteristics of the HCV-Halfzymes developed in the study, however, indicate that when used in a properly formatted assay the true HCV-Halfzyme L.O.D will meet or exceed detection of 100 molecules.

Halfzyme Technology

Enzymatic nucleic acid molecules are nucleic acid-based enzymes that, like protein

20   enzymes, accelerate biochemical reactions. Halfzymes, are a particular class of enzymatic nucleic acid molecule in which a portion of the nucleic acid sequence of the enzymatic nucleic acid molecule has been deleted and is supplied in *trans* as an effector nucleic acid. In an non-limiting example, Halfzymes are therefore devoid of catalytic activity in the absence of this effector sequence, but their activity can be induced through interaction

25   with the *trans*-acting oligonucleotide effector. In the Halfzyme system an oligonucleotide target RNA acts as an effector or activator, inducing enzymatic nucleic acid molecule catalysis (Figure 15). Consequently, Halfzyme catalytic activity acts as the readout for the presence of a particular target nucleic acid. Halfzymes function as multiple turnover

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catalysts, providing an intrinsic signal amplification step. Unlike PCR-based assays that require time consuming and difficult to automate thermal cycling, Halfzymes function in an isothermal manner. Moreover, the use of Halfzymes for nucleic acid detection does not require amplification of the target sequence, such that contamination problems

5   associated with PCR-based assays are eliminated. Halfzyme enzymatic nucleic acid molecules are assembled from two or more separate nucleic acid molecules, preferably two or three separate nucleic acid molecules.

Halfzyme technology is based on, for example, an enzymatic nucleic acid that catalyzes ligation of separate molecule, for example a class I ligase enzymatic nucleic

10   acid molecule (Figure 16). This enzymatic nucleic acid molecule motif was chosen as the catalytic 'platform' for Halfzyme ligase development because it displays one of the fastest catalytic rates described for a enzymatic nucleic acid molecule (up to 300 turnovers per minute, and has been extensively characterized. In contrast to conventional hybridization-based nucleic acid detection strategies that require stretches of twenty or more highly

15   conserved nucleotides, Halfzyme technology is designed so that target detection requires recognition of no more than nine contiguous nucleotides of conserved sequence (see for example Figure 16).

. The Halfzymes in this example covalently ligate two substrate (reporter) nucleic acids (Figure 16). In the nomenclature used by applicant, the 5' substrate RNA that

20   supplies the nucleophilic hydroxyl in the ligation reaction is referred to as substrate 2. The 3' substrate RNA that carries the 5'-triphosphate and hence the pyrophosphate leaving group is referred to as substrate 1. Each of these two substrates can be made by solid-phase oligonucleotide synthesis. Therefore, each substrate RNA can be independently and site specifically labeled with one or several haptens or capture

25   reagents. Consequently, ligated products that are amplified from Halfzyme reactions can be detected in a number of different assay formats.

<u>Hepatitis C Virus (HCV) target site</u>

The use of Halfzyme technology as a method for the sensitive detection of viral sequences was tested. Halfzyme reagents were developed that are RNA sequences present

in the Hepatitis C Virus (HCV) genome as effector molecules. Such Halfzymes are referred to as HCV-activated Halfzymes or HCV-Halfzymes.

Consistent with nucleic acid testing strategies, it is preferable to select for detection of a viral nucleic acid sequence that is highly conserved among different viral strains.
5   Hepatitis C Virus (HCV) is a positive strand RNA virus of approximately nine kilobases (9 kB) that contains one large open reading frame (ORF) encoding both structural and nonstructural proteins and a large 5' untranslated region (5'-UTR), that acts as a functional RNA to direct cap-independent translation within cells. While the ORF is highly variable in nucleotide sequence, the 5'-UTR is conserved to a much greater degree.
10   Thus, the 5'-UTR represents an attractive target for nucleic acid-based detection strategies. Within the 5'-UTR, the most highly conserved feature is a structural element referred to as stem-loop IIIB. A multiple sequence alignment of the approximately 1500 GENBANK entries of HCV sequences containing stem-loop IIIB indicates that the 3' half of this sequence is almost universally conserved among HCV isolates while the 5' portion
15   varies only slightly (Figure 17). Consequently, the sequence of stem-loop IIIB was chosen as the non-limiting example of a HCV-Halfzyme effector oligonucleotide (positions 175 to 205 by conventional numbering).

Directed Molecular Evolution (DME)

Nucleic acids possess the unique property that genotype (replicatable information)
20   and phenotype (molecular function) reside in one molecule. Thus, molecules with specific functions can be directly replicated and amplified by RT-PCR. Directed Molecular Evolution, or DME, is a process for the combinatorial selection of nucleic acids in which a large collection of random sequence variants of a functional RNA are produced and subjected to selective pressure that allows rare sequence variants with enhanced
25   performance characteristics to be the separated from the bulk of the sequence variants with less robust performance (Figure 18). Formally, DME is similar to Darwinian evolution in that it entails 'survival of the fittest': many individuals (sequences) displaying different characteristics are forced to 'compete' by subjecting them to a selective pressure. Those enzymatic nucleic acid molecule sequences with the best

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performance are allowed to reproduce (DME reproduction is through RT-PCR rather than organismal reproduction as in true evolution). After amplification, the library of sequences that is enriched for functional molecules is used in subsequent cycles of DME in which the stringency of the selective pressure is progressively increased. When the

5   properties of the library of sequences as a whole display the desired functional characteristics, individual members are cloned through bacterial transformation and individually characterized. In this way, enzymatic nucleic acid molecules with properties substantially better than the starting sequence can be identified.

Applicant has used DME to develop catalytically efficient Halfzymes that are

10   activated by a predetermined effector nucleic acid: sequences of stem-loop IIIB of the HCV 5'-UTR. This work serves as a model for the production of Halfzymes that are activated by other target nucleic acids. All work performed in this example utilized the class I ligase enzymatic nucleic acid molecule as the catalytic 'platform' for the production of Halfzyme ligase catalysts. A person skilled in the art will recognize that

15   other enzymatic nucleic acid molecules can similarly be utilized for the production of Halfzyme or other multicomponent nucleic acid constructs using the teaching of this application, including nucleic acid sensor constructs capable of catalyzing different chemical reactions.

Applicant first defined the Limit of Detection (L.O.D.) of a single turnover version

20   of an HCV-activated Halfzyme. This HCV-Halfzyme promotes the ligation of a substrate RNA oligonucleotide to its own 5' terminus. Thus, it is able to perform only a single catalytic cycle; it does not provide amplification through multiple turnover catalysis.

The sequence of the HCV-Halfzyme used in these initial studies contained sequence changes to accommodate the sequence of the HCV target oligonucleotide

25   (Figure 19). The L.O.D. was established by determination of HCV-Halfzyme activity as a function of the copy number of the HCV effector sequence. Using a synthetic oligoribonucleotide representing stem-loop IIIB of the HCV 5' UTR target site, the limit of detection of this system was 6×10^7 molecules. Importantly, Halfzymes that were activated by the complete HCV 5'-UTR displayed the same L.O.D. as the synthetic

oligonucleotide if the 5'-UTR was first pre-treated with RNase H and specific DNA oligonucleotides to liberate the HCV-Halfzyme effector nucleic acid (Figure 19). Since the 5'-UTR folds into an independent structural domain within the intact the HCV genome, the ability to activation of the HCV-Halfzyme with an effector nucleic acid

5   derived from the 5'-UTR indicates that it can be activated with sequences derived by the intact HCV genome when isolated from clinical samples.

The above work demonstrated the efficacy of using Halfzyme technology to detect viral nucleic acid sequences. However, the L.O.D. of this single turnover HCV-Halfzyme may not be sufficient for use in a number of blood screening applications. Multiple

10   turnover versions of such an HCV-Halfzyme, therefore, were needed to carry out multiple catalytic cycles for signal amplification. However, the catalytic rate of the HCV-Halfzyme used in the above studies was extremely low (k_{obs} = ~5 X 10^{-4} min^{-1}). Consequently, the production of an HCV-Halfzyme with better performance characteristics was required to decrease the L.O.D. to levels appropriate for viral

15   screening. To develop a more efficient HCV-Halfzyme, Directed Molecular Evolution (DME) was performed to identify sequence variants of the HCV-Halfzyme described above with the desired performance characteristics.

Initial HCV-Halfzyme development in DME-1

The HCV-Halfzyme sequence library used for DME-1 was produced through

20   'doped' solid phase oligonucleotide synthesis of DNA by standard procedures. A total of 62 nucleotide positions in regions of the HCV-Halfzyme not directly involved in interaction with substrate RNA or hybridization to the HCV effector were mutagenized such that each consisted of non-parental sequence 30% of the time (Figure 20). As a result, each of the ~10^{10} HCV-Halfzyme sequence variants carried ~18 changes relative to

25   the parental HCV-Halfzyme. In this HCV-Halfzyme library, HCV effector hybridized to stretches of 6, 7, and 24 nucleotides.

Each cycle of DME-1 involved Halfzyme transcription, incubation with HCV effector oligonucleotide and substrate 2, fractionation and collection of substrate 2/ HCV-

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Halfzyme ligated product from non-reacted HCV-Halfzyme by gel electrophoresis, amplification of substrate 2/ HCV-Halfzyme ligated product by RT-PCR, and a second PCR to regenerate the transcriptional promoter and appropriate 5' end of the Halfzyme pool (Figure 20). Each cycle of this selection required ~ 5 days. In successive cycles of

5   DME-1 the selective pressure for faster catalytic rates was applied by decreasing the time allowed for ligation of substrate 2 to the HCV-Halfzyme sequence library. Initially, this reaction time was 16 h, but by the seventh and final round the incubation time was reduced to 15 seconds.

       At each cycle of DME, HCV-Halfzymes with progressively faster catalytic rates

10   were enriched (data not shown). DME-1 was terminated after seven cycles of DME-1, at which point the HCV-Halfzyme library displayed a single turnover rate of 0.03 min^{-1}. This rate is similar to that obtained in the initial selection of this ligase enzymatic nucleic acid molecule. DNA representing individual sequences contained in this enriched HCV-Halfzyme library were ligated into bacterial plasmids and isolated by transformation into

15   E. coli. Individual HCV-Halfzymes were then characterized as described below.

Characterization of HCV-Halfzymes Developed in DME-1

       The kinetic properties (Table II) and sequences of clones representing thirty-three HCV-Halfzymes developed in DME-1 were characterized. Significantly, several sequence variants display an observed rate of ~ 1.5 to 2 min^{-1}. These HCV-Halfzymes

20   comprised one sequence family. The optimal HCV-Halfzyme from DME-1, clone 8/7, contains 10 nucleotide changes relative to the input sequence and one nucleotide deletion (Figure 21). These changes are responsible for increasing its activity in auto-ligation (single turnover) reactions by ~ 400-fold. The catalytic rate of 8/7 HCV-Halfzyme was characterized in terms of its dependence on pH and Mg^{2+}. The assembly of 8/7 HCV-

25   Halfzyme into active complexes and its ability to function as a multiple turnover enzymatic nucleic acid molecule were also investigated.

Table II

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Clone #	Rate (min^{-1})
8/7	2.100
8/24	1.650
8/26	1.610
8/3	1.340
8/6	1.260
8/5	1.190
8/8	1.060
8/16	0.994
8/15	0.850
8/10	0.810
8/21	0.730
8/4	0.670
8/19	0.630
8/20	0.600
8/27	0.530
8/13	0.500
8/11	0.400
8/25	0.380
8/12	0.330
8/14	0.320
8/1	0.310
8/23	0.300
8/18	0.290
8/2	0.230
8/32	0.030
8/29	0.023
8/22	0.016
8/17	0.010
8/28	0.004
8/30	0.004
8/33	0.002
8/9	0.001

A log-linear relationship between pH and observed rate of 8/7 HCV-Halfzyme would mean that the chemical step is limiting the observed rate since the chemical step of ligation promoted by the class I ligase enzymatic nucleic acid molecule is dependent on the concentration of hydroxide ion in solution. Increasing pH, therefore, could be used to increase its catalytic activity. The observed single turnover rate of clone 8/7 HCV-Halfzyme displayed a log-linear relationship with pH until pH 7.0, at which point the rates reached a plateau at ~2 min^{-1} (Figure 22). Increasing pH further did not result in an

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increase in observed rate. Such a plateau indicates that the observed rate of this HCV-Halfzyme is limited by a conformational rearrangement proceeding at ~2 min^{-1}.

The dependence of the observed rate on Mg^{2+} was examined since Mg^{2+} is beleived
to affect the folding of enzymatic nucleic acid molecules, and therefore could affect the
5   rate-limiting conformational rearrangement of the 8/7 HCV-Halfzyme that is evidenced in
its pH profile. Indeed, the catalytic rate of the 8/7 HCV-Halfzyme increased as a function
of Mg^{2+} at all concentrations tested, indicating that the folding of 8/7 HCV-Halfzyme was
incomplete at even the highest Mg^{2+} concentration tested (500 mM, Figure 22). Thus,
the optimal HCV-Halfzyme obtained from DME-1 is limited in rate by its incomplete
10   formation of an active three-dimensional structure in a way that could not be
compensated by high concentrations of Mg^{2+}.

To examine whether the gross assembly of the 8/7 HCV-Halfzyme was the cause of
its folding deficiency, a native gel electrophoresis assay was developed to monitor RNA-RNA interactions (Figure 23). This assay was used to determine the affinity of all
15   relevant RNA-RNA interactions required for assembly and function of active HCV-Halfzyme complexes. These include the affinity of substrate 2 for the HCV-Halfzyme and
the affinity of the HCV-Halfzyme for the HCV effector oligonucleotide. These data
showed that all relevant assembly events proceeded to 100% completion at the
concentrations of substrate 2 RNA, HCV-Halfzyme and HCV effector oligonucleotide
20   used in assays (data not shown). Thus, the deficiency in folding displayed by the 8/7
HCV-Halfzyme could not be accounted for by inefficient or incomplete assembly events.

Conversion of the single turnover 8/7 HCV-Halfzyme obtained from DME-1 to a
multiple turnover version involves dissection into two parts: a 5' portion becomes a *trans*
acting substrate (substrate 1), and a 3' portion that becomes the multiple turnover HCV-
25   Halfzyme. Substrate 1 is ligated to the same substrate 2 RNA that is used in single
turnover reactions. By dissecting the HCV-Halfzyme at various internucleotide positions
several multiple turnover configurations of the clone 8/7 HCV-Halfzyme were generated
(Table III). Each directs the ligation of distinct substrate 1 RNA oligonucleotide to the
substrate 2 oligonucleotide.

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Multiple turnover configurations analogous to that described for the parental class I ligase enzymatic nucleic acid molecule had low activity (multiple turnover rates less than 10^{-5} min^{-1}, Table III). However, a configuration that utilizes pppGGA as substrate 1 showed a turnover rate of 0.004 min^{-1}. This value is approximately 200-fold lower than 5   the observed rate displayed by the single turnover clone 8/7 HCV-Halfzyme. This particular configuration for a multiple turnover HCV-Halfzyme is attractive because the uncatalyzed rate of substrate RNA ligation is minimized since the reactive groups (5'triphosphate and 3' hydroxyl of the two substrate RNAs) are not held in close proximity as they are in the unimolecular substrate RNA-substrate RNA complex used in 10   other configurations.

Table III			
Configuration	Substrate 1 Sequence (5'-3')	Rate (min-1)	SEQ ID NO
1	ppp-GGA	0.004	76
2	ppp-GGAAAUCCAAACGACUGGUAC	< 10-5	77
3	Ppp-GGAAAUCCAAACGACUGGUACAAAA	< 10-5	78
4	ppp-GGAAAUCCAAACGACUGGUACAAAAAAGACAAU	< 10-5	79
5	ppp-GGAAAUCCAAACGACUGGUACAAAAAAGACAAAU GUGUGCCCUCA	< 10-5	80
7	ppp-GGAAAUCCAAACGACUG	< 10-5	81

Secondary HCV-Halfzyme Development in DME-2

A secondary DME (DME-2) was initiated to optimize folding and further increase 15   catalytic activity of the Halfzyme constructs. DME-2 was carried out to optimize folding and further increase activity of 8/7 HCV-Halfzyme obtained from DME-1. Since the

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activity of 8/7 HCV-Halfzyme was believed to be limited by the binding of Mg^{2+}, in DME-2 HCV-Halfzymes were demanded to display increased catalytic function at reduced concentrations of Mg^{2+}.

5   In DME-2 a new library of HCV-Halfzyme sequence variants was produced based on the clone 8/7 HCV-Halfzyme obtained from DME-1. In DME-2, rather than simply re-randomizing the positions already present in the clone 8/7 HCV-Halfzyme, additional regions of random sequence were added to the existing clone 8/7 HCV-Halfzyme (Figure 24). Three different HCV-Halfzyme libraries were produced for three DME-2 processes that were performed in parallel. In two of these libraries, 30 nucleotides of random 10   sequence were inserted. In the third HCV-Halfzyme library, 26 random nucleotides replaced a four nucleotide loop within the clone 8/7 HCV-Halfzyme. Each of these three libraries consisted of ~1 X 10^{15} HCV-Halfzyme sequence variants. Importantly, all three of the HCV-Halfzyme sequence libraries used in DME-2 was 3'-truncated relative to the 8/7 HCV-Halfzyme obtained from DME-1. Thus, HCV-Halfzymes produced in DME-2 15   can contiguously hybridize to nine nucleotides of HCV sequence.

          DME-2 was carried out similar to DME-1, except that: 1) the concentration of Mg^{2+} was progressively decreased in successive rounds, and 2) each of the three libraries was subjected separately to DME-2. After each cycle of DME-2, Halfzymes with faster catalytic rates were obtained even as the concentration of Mg^{2+} was reduced (data not 20   shown). To obtain the best performing HCV-Halfzymes from these three sequence libraries, all three libraries were mixed together and subject to the final cycle of DME-2 in which they were made to compete against one another. DME-2 was terminated after this cycle at which point the *pooled* HCV-Halfzyme library displayed a single turnover rate of 0.02 min^{-1} at 1 mM Mg^{2+}.

25   Single turnover ligation rates of the enriched library of HCV-Halfzyme sequences obtained in DME-2 were determined as a function of Mg^{2+} concentration and compared to the same data set obtained for the 8/7 HCV-Halfzyme obtained from DME-1. At all Mg^{2+} concentrations tested, the rates of ligation promoted by the HCV-Halfzyme library obtained from DME-2 were dramatically higher than those displayed by the 8/7 HCV-

Halfzyme from DME-1 (Figure 25). Indeed, observed rates of the HCV-Halfzyme library obtained from DME-2 were too fast to measure at Mg^{2+} concentrations above 30 mM (> 12 min^{-1}, **Figure 25**.

To directly assess ability of the HCV-Halfzymes from DME-2 to function in multiple turnover format, the rate of ligation of substrate 2 to pppGGA was tested using an appropriately 5'-truncated HCV-Halfzyme library. An improvement of at least 200-fold in this reaction was observed relative to the activity of 8/7 HCV-Halfzyme (Figure 25), suggesting that this sequence library contained HCV-Halfzymes that could efficiently function in multiple turnover format. Thus, characterization of individual HCV-Halfzymes from this library was undertaken. As in DME-1, DNA representing individual sequences obtained from DME-2 were ligated into bacterial plasmids, isolated by transformation into *E. coli* and individual HCV-Halfzymes characterized as described below.

<u>Characterization of HCV-Halfzymes from DME-2</u>

Eighty clones from the final DME-2 library were transcribed and their single turnover rates characterized under sub-optimal conditions (so that their rates would be slowed enough to quantify relative to the rate displayed by the complete library of HCV-Halfzymes). Twenty-one of these clones displayed a rate greater than the final selected pool. Nineteen of these twenty-one clones were sequenced and shown to comprise one family of related sequences. The seven displaying the best performance in this initial kinetic screen were characterized in more detail at pH 7.5, 3 mM Mg^{2+} (Table IV). While the catalytic rates of these seven clones were rather tightly clustered, clones 21 and 38 displayed the fastest rates and proceeded to 86% and 74% completion, respectively, under these conditions. Note that the plateau value in single turnover conditions can be used to judge the fraction of the HCV-Halfzyme that is active for ligation. These criteria were used to establish clone 38 and clone 21 HCV-Halfzymes as our lead reagents. Both HCV-Halfzymes display a rate of ligation that is equal to that reported for the class I constitutive ligase from which they were derived, even though the latter was measured at an even higher Mg^{2+} concentration (Table, IV). Under optimal conditions (pH 7.5, 60

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mM Mg^{2+}) both of these clones displayed single turnover rates that were >15 min^{-1} (too fast to measure by manual pipeting methods, data not shown).

Table IV

Clone #	k_{obs} (min^{-1}), Plateau
38*	4.1, 86%
21*	3.6, 74%
26*	3.3, 85%
30*	3.1, 85%
17*	3.0, 84%
35*	2.5, 88%
24*	2.1, 79%
constitutive ligase[1]	3.9, 70%
*pH 7.5, 3.0 mM Mg^{2+} [1]pH 7.5, 10.0 mM Mg^{2+}	

Clones 21 and 38 are closely related in sequence and predicted secondary structure
5   (Figure 26). In both HCV-Halfzymes, the sequence selected from the random sequence library served to shift, or slide, the base paired region (referred to as P3) 3' of its original location, dramatically changing the orientation of the highly conserved unpaired nucleotides (designated S2 and S3) thought to comprise the catalytic core of the class I enzymatic nucleic acid molecule motif.

10   Multiple turnover HCV-Halfzymes

Several multiple turnover configurations of clone 38 and clone 21 HCV-Halfzymes were developed by 5' truncation at various positions. However, work focused on two (referred to as configuration 1 and configuration 3, Figure 27). Each multiple turnover configuration requires an HCV-Halfzyme that is uniquely 5'- truncated and a substrate 1
15   of a different length. The properties of each of these configurations are quite distinct and each is described separately below.

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Characterization of Configuration 3

This configuration of multiple turnover HCV-Halfzyme was produced by 5'-truncating 23 nucleotides from the 5' end of the single turnover version of clone 21 HCV-Halfzyme. This same sequence functions in *trans* as substrate 1 (Figure 27). In
5 configuration 3, the two substrates base pair with one another to form a unimolecular complex independent of the HCV-Halfzyme. This substrate RNA complex associates with *effector-bound* HCV-Halfzyme through Watson-Crick base interaction with *effector* nucleic acid sequence. Thus, the substrate RNA complex will not associate with HCV-Halfzyme in the absence of the HCV effector nucleic acid.

10 Configuration 3 is very similar to the multiple turnover version of the class I ligase enzymatic nucleic acid molecule. To date, the maximal turnover rate of clone 38 or clone 21 HCV-Halfzyme in this configuration is 0.75 min^{-1} (assayed at pH 7.5, 60 mM Mg^{2+} and 12.5 uM substrate RNAs). This rate is approximately 20-fold lower than the rate of the single turnover reaction promoted by clone 21 HCV-Halfzyme under identical
15 conditions (>15 min^{-1}). Additional optimizations are carried so that the multiple turnover rate matches the single turnover rate, which will proportionally decrease the L.O.D. afforded by this configuration of clone 21 HCV-Halfzyme.

The native gel electrophoresis system previously described (see Characterization of HCV-Halfzymes obtained through DME-1 and Materials and Methods) was used to
20 investigate the substrate RNA concentrations required for efficient association, and the concentration of HCV-Halfzyme required for complete capture of the HCV effector oligonucleotide. This analysis showed that substrate 1 and substrate 2 have an affinity for one another of 5 nM (data not shown). The affinity of the product of ligation (produced synthetically) for HCV-Halfzyme bound to HCV effector was 1.3 uM (data not shown).
25 As expected, the simulated product did not show any interaction with HCV-Halfzyme in the absence of effector. The affinity of the HCV-Halfzyme for the HCV-effector nucleic acid was 48 nM. Complete capture of effector by HCV-Halfzyme occurred at 500 nM and set the concentration of HCV-Halfzyme used in L.O.D. determinations. Complete

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saturation of substrate RNA complex to the effector bound HCV-Halfzyme was achieved at concentrations of ~13 uM.

Optimization of Configuration 3 for 32P L.O.D. Determinations

Conditions were optimized for determining the L.O.D. of clone 21 HCV-Halfzyme

5   in configuration 3. The assay for L.O.D. determination utilizes substrate RNA that is partially labeled with ^{32}P, gel electrophoresis to resolve ligated product from substrate, and phosphoimage analysis for the direct visualization and quantification of ligated product. Because the L.O.D. determinations were performed with partially labeled substrate RNA, maximization of *detectable* signal required substrate RNA concentrations

10   that did not support fully catalytic activity of the HCV-Halfzyme. Thus, in the assay performed not every cycle of HCV-Halfzyme catalysis can be monitored in the L.O.D. determinations.

To optimize signal in the L.O.D. determinations using the ^{32}P assay, HCV-Halfzyme signal in the presence of 1 X 10^7 effector molecules was investigated as a

15   function of pH, substrate RNA concentration and Mg^{2+} concentration (Figure 28).

Examination of the dependence of signal and turnover rate on pH and substrate concentration shows that clone 21 HCV-Halfzyme turnover rate and detectable signal increases 5-fold from pH 6.5 to 7.5 (5-fold increase), but begins to plateau at higher pH values (Figure 28A,B). As expected, signal increased at every pH as substrate RNA was

20   decreased (Figure 28A). However, turnover rate decreased (Figure 28B). To more closely examine the effect of lowering substrate RNA concentration on signal and turnover rate a second optimization was conducted at lower substrate RNA concentrations (Figure 28C,D). This optimization was performed as a matrix with varying Mg^{2+} concentration since the affinity of the substrate RNA complex for clone 21

25   HCV-Halfzyme could be influenced by Mg^{2+} concentration. As expected, these data showed that signal increased as the substrate RNA concentration decreased (Figure 28 C,D). Maximal signal was obtained at 60 to 120 mM Mg^{2+}. This analysis was used to establish the following conditions for L.O.D. determinations using partially radiolabeled

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substrate RNA: pH 7.5, 60 mM Mg^{2+}. L.O.D. determinations were performed at several substrate RNA concentrations. Additional trials to more finely define the optimal conditions for L.O.D. determinations are carried out to fully optimize assays using the ^{32}P-based assay.

5   Since pH effects HCV-Halfzyme activity (and consequently L.O.D.), the dependence of the ligation rate on pH was analyzed in the presence and absence of HCV effector. The resultant curves show very different profiles (Figure 29). The maximal rate difference between + effector and – effector ligation rates occurs between pH 7.5 and 6.5. The L.O.D. determinations reported below were carried out at pH 7.5; at this pH the rate
10   differential between in the presence and absence of effector is 6.2 X 10^7.

All L.O.D. determinations were carried out at n=5. Positive signal was judged as an average signal from the 5 trials that was greater than 2 standard deviations above the signal seen in the absence of HCV effector (2SD L.O.D.). Trials were conducted at pH 7.5, 60 mM Mg2+, 0.5 to 0.1 uM clone 21 HCV-Halfzyme and 0.5 uM substrates 1 and 2.
15   Under these conditions, the average 2SD L.O.D. of the clone 21 HCV-Halfzyme in configuration 3 is 1,800 molecules (Figure 30). In all of the HCV-Halfzyme L.O.D. determinations performed, fluctuations in system background, the level of uncatalyzed ligation of the substrate RNAs, and assay operator variability from experiment to experiment allowed the 2SD L.O.D. to range from 600 molecules to 18,000 molecules.
20   Detectable signal above background was observed as low as 60 molecules. Coefficient of Variance (CV) values in L.O.D. determinations typically ranged from 10 to 20%.

Several characteristics of the assay performed reduce or limit assay sensitivity and increase CV. Due to the signal detection capabilities of the phosphoimager instrument used to quantify signal and the use of partially labeled substrate RNA, Halfzyme reactions
25   were carried out for long incubation times (usually 18 hours or longer). During such long incubation times, a loss of Halfzyme activity is expected. Uncatalyzed, 'background' ligation due to the intrinsic chemical reactivity of the two substrates is constant during this incubation period. Consequently the ratio of signal (+ effector Halfzyme catalysis) to noise (uncatalyzed background ligation) increases when incubation times are increased.

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Further, to assess the amount of ligated product RNA, it was separated from substrate RNA based on its mobility in gel electrophoresis. Due to the nature of gel electrophoresis, a small amount of the substrate RNA always "bleeds" into the position where the ligated product migrates. This "bleeding" creates a background of radioactivity and impacts our

5   ability to visualize ligated product. Detection strategies that utilize completely labeled substrate RNA and that are more sensitive than phosphoimage analysis are likely to result in a decrease in the L.O.D. afforded by Halfzyme technology.

Configuration 1

In contrast to configuration 3, HCV-Halfzyme in configuration 1 utilizes a tri-

10   nucleotide substrate 1 that does not base pair to substrate 2, and interacts with the HCV-Halfzyme largely through non-Watson-Crick interactions. In addition, because substrate 1 and substrate 2 do not interact with each other in the absence of HCV-Halfzyme in configuration 1, uncatalyzed "background" ligation is minimized (see below). This configuration of multiple turnover HCV-Halfzyme was produced by deleting 4

15   nucleotides from the 5' end of the single turnover version of clone 38 HCV-Halfzyme. 5'-pppGGA was supplied in *trans* as substrate 1 (Figure 31).

Product release is the rate-limiting step for isothermal, multiple turnover, HCV-Halfzyme configuration 1 catalysis (when the standard substrate 2 is used). To increase the rate of product dissociation, a series of 5' truncated substrate 2 RNAs were assayed

20   for their ability to promote multiple turnover catalysis (Table V). When the interaction between substrate 2 and clone 38 HCV-Halfzyme was reduced to three Watson-Crick base pairs, the multiple turnover rate at room temperature nearly matched the rate measured for the first turnover (0.113 min^{-1} vs 0.4 min^{-1}, respectively). Michaelis-Menten analysis was used to establish the affinity of several of the different sequence versions of

25   substrate 2 for the HCV-Halfzyme. This analysis suggests that the best performing substrate 2, substrate 2-4a, has an affinity of ~11 uM for the clone 38 HCV-Halfzyme. The rate provided by saturating concentrations of substrate 2-4a is ~ 150 fold reduced relative to the autoligation event promoted by this HCV-Halfzyme. In part, this reduction in rate is due to the use of sub-saturating concentrations of pppGGA (substrate 1). As

with configuration 3, additional optimization is carried out so that the multiple turnover rate afforded by configuration 1 matches the autoligation rate. In this regard, the affinity of the pppGGA substrate, or variants thereof, is increased experimentally for the HCV-Halfzyme.

Table V

Substrate 2	Sequence¶	kobs (min-1)	KRNA (µM)
standard sub	aaACCAGUC	0.0004¥	
substrate 2-1	CCAGUC	0.006§	
substrate 2-2	CAGUC	0.056§	
substrate 2-3	AGUC	0.086	12
substrate 2-4	GUC	0.059§	
substrate 2-3a	aAGUC	0.087£	15
substrate 2-4b	uAGUC	0.080£	15
substrate 2-4c	uaaAGUC	0.072£	15
substrate 2-4a	aauGUC	0.113£	11

¶lower case letters denote nucleotides that do not base pair with HCV-Halfzyme but act to destabilize the interaction of substrate 2 anf HCV-Halfzyme.
¥observed rate in presence of 20 µM substrate RNA
§observed rate in presence of 200 µM substrate RNA
£true multiple turnover rates derived from Michaelis-Menten analysis

A L.O.D. lower than 1×10^6 HCV molecules was established using the following conditions: 4 mM pppGGA, 0.5 uM HCV-Halfzyme, pH 8, and <100 nM substrate 2-4a. Importantly, in reactions that lacked HCV-effector but were otherwise identical, no ligation could be detected after an incubation of 110 hours. Therefore, the rate of ligation in the absence of effector in configuration 1 must be less than 3×10^{-9} min^{-1} (at pH 8). Given that the HCV-Halfzyme in the presence of effector has a rate of 0.113 min^{-1}, this gives a +effector/-effector rate differential greater than 3.8×10^7. This rate differential is *at least* equal to the + effector/-effector rate differential afforded by configuration 3.

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Consequently, in so far as the rate differential controls L.O.D., the true L.O.D. afforded by configuration 1 is predicted to be as good or better than the L.O.D. provided by configuration 3. Thus, the very low extent of 'background' RNA ligation suggests that multiple turnover HCV-Halfzymes in configuration 1, or variants of configuration 1, may ultimately provide more sensitive detection of nucleic acids than Halfzymes in multiple turnover configuration 3.

HCV-Halfzyme Development: Conclusions

Applicant has developed HCV-activated Halfzymes and to determine their Limit of Detection (L.O.D.). This study entailed the production of HCV-Halfzymes derived from successive Directed Molecular Evolution processes, their biochemical characterization, the construction of multiple turnover HCV-Halfzymes, and their use in limit of detection (L.O.D.) determinations.

The HCV-Halfzymes that were ultimately obtained from DME display autoligation rates (ligation of substrate 2 to their own 5' end) that are indistinguishable from the constitutively active class I ligase upon which they are based. The observed rate of this reaction is too fast to measure under the conditions used for L.O.D. determinations (>15 min^{-1}), and could equal class I ligase (~120 min^{-1} under these conditions). Thus, DME produced HCV-Halfzymes that were extremely efficient at performing autoligation reactions.

Several different configurations of multiple turnover HCV-Halfzymes were developed from the efficient HCV-Halfzymes obtained in DME. Of these, two configurations (1 and 3) were characterized in detail. The two configurations have very different properties. In configuration 3, the two substrates interact with one another through Watson-Crick interactions in the absence of HCV-Halfzyme. These two substrates associate with the effector bound HCV-Halfzyme by forming Watson-Crick base pairs with the HCV effector. In configuration 1, the two substrates do not interact with one another. Substrate 2, but not substrate 1, forms a stable complex with the HCV-Halfzyme.

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The multiple turnover rates of these HCV-Halfzyme configurations are 0.75 min^{-1} (configuration 3) and 0.1 min^{-1} (configuration 1). Thus, the time required for single catalytic cycles in the two configurations is 1.4 minutes and 10 minutes, respectively. Optimization are to maximize these multiple turnover rates was underway. Multiple turnover HCV-Halfzyme catalysts that function with a rate identical to the autoligation reaction would produce >15 products per minute.

These two configurations of multiple turnover HCV-Halfzyme differ dramatically in the rate of background ligation that they promote. In configuration 3, this rate is 3.2 X 10^{-9} min^{-1} at the conditions used for L.O.D. determinations. In contrast, the substrate RNAs used for HCV-Halfzymes in configuration 1 show absolutely no background ligation (indicating a rate of no more than 1 X 10^{-X} min^{-1}).

L.O.D. determinations conducted at RPI utilized substrate RNAs that were partially labeled with 32P, gel electrophoresis to resolve ligated product from substrate, and phosphorimage analysis for the direct visualization and quantification of ligated product. Using conditions that optimize detectable signal rather than turnover rate, the configuration 3 HCV-Halfzyme yielded an average 2SD L.O.D. of 1800 molecules.

Materials and Methods

RNA Synthesis

Substrate 2 RNAs were produced through standard oligoribonucleotide synthesis procedures. 5' triphosphorylated substrate 1 oligoribonucleotides were made either by in vitro T7 RNA polymerase transcription of a corresponding DNA template, or by organic synthesis (procedure described below). Halfzymes were produced by in vitro T7 RNA polymerase transcription from DNA templates. DNA templates were either generated from PCR of an existing Halfzyme construct, or from two overlapping anti-parallel DNA oligonucleotides that were first extended to completion with Taq polymerase.

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Preparation of 5'-triphosphyrlated RNA

Oligonucleotide 5'-triphosphates were prepared by subjecting solid support bound oligonucleotide to the conditions used for the preparation of nucleoside 5'-triphosphate (19). Modifications to this procedure (described below) were introduced in order to make

5   it suitable for synthesis on oligoribonucleotides attached a to solid support.

Organic Synthesis:

1. Dry 2.5 uM synthesis column at 35 °C under high vacuum for 2 h.
2. Wash column with dry pyridine (10 mL) followed by dry DMF (10 mL).
3. Slowly push through the column 2 mL of freshly prepared solution of salicyl
10   chlorophosphite (0.81g) in dioxane-pyridine-DMF (2.5:1:0.5, 4 mL) for 8 min. Discard the solution and repeat the above procedure with 2 mL fresh of reagent. Total time:16 min.
4. Wash column with dioxane (10 mL), followed by acetonitrile (10 mL).
5. Slowly push through the column 2 mL of well mixed 0.5 M $P_2O_7^{4-} \cdot 1.5$ Bu$_3$N
15   (Sigma, 0.712 g) in DMF-Bu$_3$N (3:1, 4 mL) for 10 min. Discard the solution and repeat the above procedure with fresh 2 mL of reagent. Total time – 20 min.
6. Wash column with DMF (10 mL), followed by acetonitrile (10 mL).
7. Push through the column 2 mL of oxidation solution (3g I$_2$ in H$_2$O-pyridine-THF 2:20:75) for 20 min.
20   8. Wash column with 70% pyridine-water (10 mL), acetonitrile (2x10 mL) and THF (10 mL). Dry with air or under vacuum.

Deprotection:

Base deprotection: 2 mL of conc. ammonia, 5 h @ 60 oC..

25   TBDMS cleavage: 2 mL of 1M TBAF (Aldrich) (dried for 3 days over activated 4A molecular sieves), 16 h. Quench with 5 mL 1.5 M sodium acetate (pH 5.2). THF removed in vacuo, aq. layer extracted twice with ethyl acetate. Precipitation of the product with 20 mL of ethanol, followed by centrifugation at 16000 x g produced a pellet.

Gel Electrophoresis

Denaturing Gel Electrophoresis

Denaturing, 20% acrylamide gels (19:1 acrylamide:bis-acrylamide) were run at room temperature at a constant power of 50 Watts for approximately 3 h in 1X TBE buffer (90mM Tris-Borate, 4mM EDTA). Gels were pre-run for approximately for 0.5 h before loading the samples in an equal amount of gel loading dye (95% formamide, 10mM EDTA, 0.003% bromophenol blue and xylene cyanol). After running and disassembly, gels were dried and used to expose Molecular Dynamics Phosphoimager cassettes. The intensity of radiolabeled RNA was determined using Imagequant software (Molecular Dynamics).

Non-denaturing Gel Electrophoresis and RNA-RNA Affinity Determinations

Ten percent non-denaturing acrylamide gels (19:1 acrylamide:bis-acrylamide) were run at a constant power of 50 Watts for approximately 5 h and used the following buffer conditions: 50mM Tris-HCl pH 7.5, 0.6mM EDTA, 30 or 60mM MgCl2. The temperature of the gel was held constant at 23°C by using an antifreeze coolant coil placed in the buffer chamber adjacent to the gel and the pH was maintained at 7.5 by constant circulation of the buffer between the upper and lower chambers of the gel apparatus using a peristaltic pump. In each experiment, one of the two RNAs was 5'-end radiolabeled and used in trace amounts while the second RNA varied in concentration. Binding reactions were allowed to equilibrate and loaded directly onto pre-assembled native gels. After running and disassembly, gels were used to expose Molecular Dynamics Phosphoimager cassettes. The intensity of complexed and uncomplexed radiolabeled RNA was determined using Imagequant software (Molecular Dynamics). The affinity of RNA-RNA interactions was determined using KaleidaGraph software and data fit to the equation: fraction bound=[non-labeled RNA]/(KD[non-labeled RNA]), where [non-labeled RNA] represents the RNA that varied in concentration.

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Apparatus Set Up

Two plates of glasses, spacers and a comb were wiped by 95% EtOH. 1/150 volume of 10% APS and 1/1500 volume of TEMED were added to x% acrylamide in 7M Urea-1xTBE. After mixing those, the acrylamide solution was thrown into the glass

5   plates as soon as possible, and the comb was put into. 30-minutes later, the comb was taken off and the gel was pre-run by 1X TBE. After pre-running, samples, which were mixed loading dye (e.g. 9 5% Formamide, 10mM EDTA, 0.03% BPB, 0.03% XP), were applied on the gel. After running, a gel was quantitated by phosphoimage analysis. For example, positions of substrate 2 and product are as a right picture on 15% acrylamide

10   gel.

Kinetic Assays

Kinetic assays were performed at 23oC in 30mM buffer at 3 mM to 120 mM MgCl2 as specified. Solutions were buffered with MES (pH 5.5, 6.0, 6.5) or Tris-HCl (pH 7.0, 7.5, 8.0, 8.5, 9.0). In all assays, the HCV-Halfzyme and effector were heated at

15   80oC for two minutes, 5X buffer was immediately added, and the reaction allowed to cool to 23oC over 5 minutes. Reactions were initiated by addition of substrate RNAs.

Single turnover kinetic assays

A trace concentration of 5'-32P labeled Substrate 2 (<5nM) was incubated with 1 uM HCV-Halfzyme. Time points were taken from 5 sec. to 30 minutes depending on the

20   catalytic rate of the HCV-Halfzyme. Single turnover observed rates were determined by fitting the quantified data either to a linear equation (fraction ligated versus time) or to the single exponential equation: fraction ligated = Fa(1-e-kt), where t equals time, k equals rate of catalysis, and Fa equals the fraction of ligated substrate 2 at completion. Data was fit using Kalidagraph (Synergy Software).

Multiple turnover kinetic assays

Turnover rates were calculated from the initial rate of the reaction (<20% substrate converted to ligated product) and fit to the following equation: ([ligated product]/[32P substrate 2]*[HCV-Halfzyme]). When required, Michaelis-Menten parameters were established by varying substrate concentration and fitting to the Michaelis-Menten equation: Data were fit to the equation: $v = [E][S]kcat/(KM+[S])$, where v equals rate at each [S], S represents substrate 1 concentration (the concentration of substrate 2 was always equal to substrate 1 concentration), KM equals apparent binding constant for half-maximal activation. Conditions for the different configurations of multiple turnover HCV-Halfzymes are described below.

Table VI

	+Effector	-Effector	No HZ, No Eff	Final conc.	
Halfzyme (5uM)	2	2	-	0.5uM	Solution 1
Effector (5uM)	2	-	-	0.5uM	
H₂O	1.9	3.9	5.9		
Heat 80 degree for 2mins., cool to 22 degree for 5 mins.					
5 x reaction buffer	4	4	4	1x	
substrate 1	5	5	5		Solution 2
substrate 2	5	5	5		
³²P-substrate 2	0.1	0.1	0.1	Trace	
Total	20	20	20		
	(ul)	(ul)	(ul)		

Configuration 1: Unless otherwise noted, assays were carried out at 4 mM pppGGA, <5 nM to 25 uM of 5'-32P labeled substrate 2, 0.5uM HCV-Halfzyme, 40 mM MgCl2, and 30 mM Tris-HCl (pH 8.0). Data points were taken from 1h to 48h.

Configuration 3: Assays were carried out with equal concentrations of substrate 1 and substrate 2 (at 0.5 uM), trace 5'-32P labeled substrate 2, 0.5 HCV-Halfzyme, 60 or 120 mM MgCl2 at pH 6.5 or 7.5. Data points were taken from 0.5 h to 20 h.

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Limit of Detection (L.O.D.) Determinations

L.O.D. determinations were carried out at 23°C in 30mM buffer, 0.6mM EDTA, and at different pHs and concentrations of MgCl2 depending on the particular configuration of multiple turnover HCV-Halfzyme (see below). Assays contained 0, 60, 180, 600, 1800, 6000, 1.8 X 104, 1.8 X 105, 1.8 X 106 or 1.8 X 107 HCV effector molecules, which were serially diluted into 100ng/μL yeast tRNA from a concentrated stock solution of synthetic HCV-effector oligonucleotide (concentration determined by OD260 and the extinction coefficient of the HCV effector oligonucleotide, ε = 2.8324 X 105 M-1 cm-1).

In all assays, HCV-Halfzyme (0.5 uM) and effector nucleic acid were heated together at 80oC for two minutes, 5X buffer immediately added, and allowed to cool to 23oC over 5 minutes. Reactions were initiated by addition of both substrate RNAs. When the level of HCV effector approached the L.O.D. of the HCV-Halfzyme reactions were performed at n = 5. Control reactions in which HCV effector nucleic acid was omitted were also performed at n = 5.

Configuration 1: Assays were carried out at 1 mM pppGGA, 12.5 uM Substrate 2-4a, 0.5uM HCV-Halfzyme, effector, 30mM Tris-HCl (pH=8.0), 120 mM MgCl2, and 0.6mM EDTA. Reaction time was 65 h.

Configuration 3: Assays were carried out at 0.5uM of Substrate 1 and Substrate 2, 0.5 uM HCV-Halfzyme, effector, 30mM Tris-HCl (pH=7.5), 60mM MgCl2, and 0.6mM EDTA. Reaction time was 45h.

Table VII

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	+Effector	-Effector	Final conc.	
Halfzyme (5uM)	0.25	0.25	0.5uM	Solution 1
Effector	1	-		
tRNA (100ng/ul)	-	1		
H₂O	1.55	2.55		
Heat 80 degree for 2mins., cool to 22 degree for 5 mins.				
5 x reaction buffer	1	1	1x	
substrate 1	0.5	0.5		Solution 2
substrate 2	0.5	0.5		
³²P-substrate 2	0.2	0.2		
Total	5	5		
	(ul)	(ul)		

Directed Molecular Evolution

DME-1:

Library construction:

The pool for DME-1 was derived from the Class-1 ligase. The pool contained a central region of 62 positions mutagenized to 30% and flanked on both sides by constant sequence region (5'-ACACCGGAATTGCCAGGACGACCGgggggtgcctcccctggatccgaagatcggtccttgctctgaggg cacatttgtcttttacgGTACCAGTCGTTTGGATTTCC-3') (SEQ ID NO: 82). The pool was amplified in a 5 mL PCR reaction using primers that added the promoter sequence for T7 RNA polymerase (5'-GCTAATACGACTCACTATAGGAAATCCAAACGACTGGTAC-3' (SEQ ID NO: 83) and 5'-ACACCGGAATTGCCAGG-3', (SEQ ID NO: 84)). The final complexity of the population was 1X10¹⁰. One nmole of the pool DNA was transcribed with T7 polymerase in a 2 mL reaction and RNA purified on a 10% polyacrylamide gel.

Selection:

Selection was carried out starting with 2X10¹⁵ molecules (4 nmoles). Pool RNA and 1.1 equivalent of effector RNA (5'-ACACCGGAAUUGCCAGGACGACCGGGUCCUUUCUUGGAUAA-3', (SEQ ID NO: 85)) was heated in water to 80oC for 3 min. and cooled to room temperature for 10 min. 2X selection buffer was added (final buffer conditions: 30 mM Tris (pH 7.5), 200 mM

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KCl, 0.6 mM EDTA and 60 mM MgCl2) along with 2.2 equivalents of a substrate 2 variant that allowed ligation product-specific PCR. After a define period of incubation, the reaction was stopped by the addition of EDTA, HCV-Halfzyme ligated to substrate 2 was purified on a 10% denaturing acrylamide gel. The selected RNA was amplified by

5   RT-PCR and the T7 promoter was restored through a nested PCR. A total of 8 rounds of DME were performed. At each, the selection stringency was increased by progressively decreasing the ligation time from 16 h to 15 sec. in round 8th. A negative selection step of 20h incubation in the absence of effector of 20 h was introduced in round 4th to prevent the amplification of effector-independent ligases.

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DME-2:

Library construction:

The pools for DME-2 were constructed based on the 8/7 HCV-Halfzyme from DME-1. Three libraries were constructed in which random sequences of either 30 or 26

15   nucleotides   were   inserted   at   different   positions   (library-1,   5'-CCAGGACGACTGCAGGGTGCCACCTGTAGATC(N30)GATCGGTCCTTGATCTG AGGGCACATTTGTCTTTTTTG-3'   (SEQ   ID   NO: 86);   library-2,   5'-GGAAATCCAAACGACTGGTACAAAAAAGACAAAT(N26)GTGCCCTCAGATCA AGGACCGATCTTCGGATCTACAGG-3'   (SEQ   ID   NO: 87);   library-3,   5'-

20   GGAAATCCAAACGACTGGTACAA(N26)AAAAGACAAATGTGCCCTCAGATCA AGGACCGATCTTCGGATCTACAGG-3',   (SEQ   ID   NO: 88)).   Each   library   was amplified in a 5 mL PCR reaction using primers that extended the 5' constant region and added   the   promoter   sequence   for   T7   RNA   polymerase   (5'-GCTAATACGACTCACTATAGGAAATCCAAACGACTGGTACAAAAAAGACAA

25   ATGTGCC-3',   (SEQ   ID   NO: 89);   5'-CCAGGACGACTGCAGGGTGCCACCTGTAGATCCGAAGATCGGTCC-3', (SEQ ID NO: 90); 5'-GCTAATACGACTCACTATAGGAAATCCAAACGACTGGTAC-3' (SEQ ID NO: 91) and 5'-CCAGGACGACTGCAGGGTGCC-3', (SEQ ID NO: 92)). The final complexity of the each population was ~3X1014. 0.6 nanomole of the each pool was

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transcribed separately with T7 RNA polymerase in a 1 mL reaction and RNA purified on a 10% polyacrylamide gel.

Selection:

Selection was carried out starting with 1×10^{15} molecules for each DME. Each library was subjected to selection at pH 6.0 (MES) and pH 7.5 (Tris-HCl). Library RNA and 1.1 equivalent of effector RNA (5'-CCAGGACGACCGGGUCCUUUCUUGGAUAA-3', (SEQ ID NO: 93)) was heated in water to 80oC for 3 min. 4X selection buffer was immediately added along with MgCl2 to bring the buffer conditions at 30 mM Tris pH 7.5 (or MES pH 6.0), 0.6 mM EDTA and 0.1% NP40. A total of 8 rounds of DME were performed. MgCl2 was progressively decreased to increase the stringency of selection (20 mM in round 1 to 3 mM in round 8). Selection stringency was also increased by progressively decreasing the ligation time from 10 min. to 5 sec. in the round 8th. Reactions were started by the addition of substrate 2. Selected RNA was amplified as in DME-1. A negative selection step of 20 h was introduced in rounds 5, 7 and 8 to prevent the amplification of effector-independent ligases. Libraries from DME-2s conducted at pH 6 and pH 7.5 were mixed separately after round 7 and made to compete against one another.

Cloning and Sequencing

To identify individual clones, DNA from the final cycle of DME was cloned into E. coli (TOP10) using TOPO TA cloning kit according to manufacturer's instructions (Invitrogen). Cloned DNA from individual colonies was amplified by the colony PCR method using M13 forward and M13 reverse primers. Both strands of each clone were PCR sequenced using dideoxy-terminated sequencing and fluorescent dyes (ABI). Sequencing reactions were analyzed on an ABI Prism 310 Genetic Analyzer and sequence alignments performed using DS Gene software.

Other uses

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The nucleic acid sensor molecules of this invention can be used as diagnostic tools to examine genetic drift and mutations within diseased cells or to detect the presence of a specific RNA in a cell. The close relationship between nucleic acid sensor molecule activity and the structure of the target RNA allows the detection of mutations in any region of the molecule which alters the base-pairing and three-dimensional structure of the target RNA. By using multiple nucleic acid sensor molecules described in this invention, one can map nucleotide changes which are important to RNA structure and function *in vitro*, as well as in cells and tissues. Cleavage of target RNAs with nucleic acid sensor molecules can be used to inhibit gene expression and define the role (essentially) of specified gene products in the progression of disease. In this manner, other genetic targets can be defined as important mediators of the disease. These experiments can lead to better treatment of the disease progression by affording the possibility of combinational therapies (*e.g.*, multiple nucleic acid sensor molecules targeted to different genes, nucleic acid target molecules coupled with known small molecule inhibitors, or intermittent treatment with combinations of nucleic acid sensor molecules and/or other chemical or biological molecules). Other *in vitro* uses of nucleic acid sensor molecules of this invention comprise detection of the presence of mRNAs associated with a disease-related condition. Such RNA is detected by determining the presence of a cleavage product after treatment with an enzymatic nucleic acid molecule using standard methodology.

In a specific example, nucleic acid sensor molecules which cleave only wild-type or mutant forms of the target RNA are used for the assay. The first nucleic acid sensor molecule is used to identify wild-type RNA present in the sample and the second nucleic acid sensor molecule is used to identify mutant RNA in the sample. As reaction controls, synthetic substrates of both wild-type and mutant RNA are cleaved by both nucleic acid sensor molecules to demonstrate the relative nucleic acid sensor molecule efficiencies in the reactions and the absence of cleavage of the "non-targeted" RNA species. The cleavage products from the synthetic substrates also serve to generate size markers for the analysis of wild-type and mutant RNAs in the sample population. Thus, each analysis can require two nucleic acid sensor molecules, two substrates and one unknown sample,

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which are combined into six reactions. The presence of cleavage products is determined using an RNAse protection assay so that full-length and cleavage fragments of each RNA can be analyzed in one lane of a polyacrylamide gel. It is not required to quantify the results to gain insight into the expression of mutant RNAs and putative risk of the desired

5   phenotypic changes in target cells. The expression of mRNA whose protein product is implicated in the development of the phenotype is sufficient to establish risk. If probes of comparable specific activity are used for both transcripts, then a qualitative comparison of RNA levels is sufficient and decreases the cost of the initial diagnosis. Higher mutant form to wild-type ratios are correlated with higher risk whether RNA levels are compared

10   qualitatively or quantitatively.

Additional Uses

Potential usefulness of sequence-specific nucleic acid sensor molecules of the instant invention have many of the same applications for the study of RNA that DNA restriction endonucleases have for the study of DNA (Nathans *et al.*, 1975 *Ann. Rev.*

15   *Biochem.* 44:273). For example, the pattern of restriction fragments can be used to establish sequence relationships between two related RNAs, and large RNAs can be specifically cleaved to fragments of a size more useful for study. The ability to engineer sequence specificity of the enzymatic nucleic acid molecule is ideal for cleavage of RNAs of unknown sequence. Applicant describes the use of nucleic acid molecules to detect

20   gene expression of target genes in bacterial, microbial, fungal, viral, and eukaryotic systems including plant, or mammalian cells.

The nucleic acid sensor molecules of the invention represent a new class of therapeutic agents capable of modulating the expression of target genes, peptides, proteins, and other biologically active molecules *in vivo* as described herein. The

25   therapeutic activity of nucleic acid sensor molecules of the invention can respond to both internal and external stimuli in a subject, for example the presence of a gene, pathogen, SNP, peptide, protein, RNA, metabolite, neurotransmitter, co-factor, drug, toxin, or physical stimuli such as light, gravity, temperature, and pressure.

All patents and publications mentioned in the specification are indicative of the

30   levels of skill of those skilled in the art to which the invention pertains. All references

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cited in this disclosure are incorporated by reference to the same extent as if each reference had been incorporated by reference in its entirety individually.

One skilled in the art would readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The methods and compositions described herein as presently representative of preferred embodiments are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses which are encompassed within the spirit of the invention, are defined by the scope of the claims.

It will be readily apparent to one skilled in the art that varying substitutions and modifications can be made to the invention disclosed herein without departing from the scope and spirit of the invention. Thus, such additional embodiments are within the scope of the present invention and the following claims.

The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments, optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the description and the appended claims.

In addition, where features or aspects of the invention are described in terms of Markush groups or other grouping of alternatives, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group or other group.

Other embodiments are within the following claims.

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Table I

A. 2.5 μmol Synthesis Cycle ABI 394 Instrument

Reagent	Equivalents	Amount	Wait Time* DNA	Wait Time* 2'-O-methyl	Wait Time*RNA
Phosphoramidites	6.5	163 μL	45 sec	2.5 min	7.5 min
S-Ethyl Tetrazole	23.8	238 μL	45 sec	2.5 min	7.5 min
Acetic Anhydride	100	233 μL	5 sec	5 sec	5 sec
N-Methyl Imidazole	186	233 μL	5 sec	5 sec	5 sec
TCA	176	2.3 mL	21 sec	21 sec	21 sec
Iodine	11.2	1.7 mL	45 sec	45 sec	45 sec
Beaucage	12.9	645 μL	100 sec	300 sec	300 sec
Acetonitrile	NA	6.67 mL	NA	NA	NA

B. 0.2 μmol Synthesis Cycle ABI 394 Instrument

Reagent	Equivalents	Amount	Wait Time* DNA	Wait Time* 2'-O-methyl	Wait Time*RNA
Phosphoramidites	15	31 μL	45 sec	233 sec	465 sec
S-Ethyl Tetrazole	38.7	31 μL	45 sec	233 min	465 sec
Acetic Anhydride	655	124 μL	5 sec	5 sec	5 sec
N-Methyl Imidazole	1245	124 μL	5 sec	5 sec	5 sec
TCA	700	732 μL	10 sec	10 sec	10 sec
Iodine	20.6	244 μL	15 sec	15 sec	15 sec
Beaucage	7.7	232 μL	100 sec	300 sec	300 sec
Acetonitrile	NA	2.64 mL	NA	NA	NA

C. 0.2 μmol Synthesis Cycle 96 well Instrument

Reagent	Equivalents:DNA/ 2'-O-methyl/Ribo	Amount: DNA/2'-O-methyl/Ribo	Wait Time* DNA	Wait Time* 2'-O-methyl	Wait Time* Ribo
Phosphoramidites	22/33/66	40/60/120 μL	60 sec	180 sec	360sec
S-Ethyl Tetrazole	70/105/210	40/60/120 μL	60 sec	180 min	360 sec
Acetic Anhydride	265/265/265	50/50/50 μL	10 sec	10 sec	10 sec
N-Methyl Imidazole	502/502/502	50/50/50 μL	10 sec	10 sec	10 sec
TCA	238/475/475	250/500/500 μL	15 sec	15 sec	15 sec
Iodine	6.8/6.8/6.8	80/80/80 μL	30 sec	30 sec	30 sec
Beaucage	34/51/51	80/120/120	100 sec	200 sec	200 sec
Acetonitrile	NA	1150/1150/1150 μL	NA	NA	NA

• Wait time does not include contact time during delivery.

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Claims

1. A nucleic acid sensor molecule comprising an enzymatic nucleic acid component and a separate effector component, wherein the enzymatic nucleic acid is assembled from two or more separate nucleic acid molecules, wherein the separate effector component is one of the two or more separate nucleic acid molecules that make up the enzymatic nucleic acid component of the nucleic acid sensor molecule, such the in the presence of the separate effector component, the enzymatic nucleic acid component assembles in a form necessary to enable the nucleic acid sensor molecule to catalyze a chemical reaction involving one or more reporter molecules, and wherein the effector and the reporter molecules are separate molecules.

2. The nucleic acid sensor molecule of claim 1, wherein the chemcial reaction is a ligation reaction.

3. The nucleic acid sensor molecule of claim 2, wherein the ligation reaction involves covalent attachment of a first reporter molecule to a second reporter molecule.

4. The nucleic acid sensor molecule of claim 2, wherein the ligation reaction results in the formation of a phosphodiester bond.

5. The nucleic acid sensor molecule of claim 3, wherein the first or second reporter molecule independently comprises a terminal phosphate group.

6. The nucleic acid sensor molecule of claim 1, wherein the chemcial reaction is a phosphodiester cleavage reaction.

7. The nucleic acid sensor molecule of claim 1, wherein the reporter molecule comprises one or more polynucleotides.

8. The nucleic acid sensor molecule of claim 1, wherein the enzymatic nucleic acid component is assembled from two separate nucleic acid molecules.

9. The nucleic acid sensor molecule of claim 1, wherein the enzymatic nucleic acid component is assembled from three separate nucleic acid molecules.

10. A method, comprising:

(a) contacting the nucleic acid sensor molecule of claim 1 with a system under
5   conditions suitable for the nucleic acid sensor molecule and to catalyze a chemical reaction on a reporter molecule; and

(b) assaying for the chemical reaction on the reporter molecule.

11. The method of claim 10, wherein the chemical reaction is indicative of the presence of a target nucleic acid in the system.

10   12. The method of claim 10, wherein the chemical reaction is indicative of the system lacking a target nucleic acid.

13. The nucleic acid sensor molecule of claim 1, wherein the effector component is an RNA or DNA derived from a bacteria, virus, fungi, plant or mammalian genome.

14. The method of claim 11, wherein the target nucleic acid is an RNA or DNA derived
15   from a bacteria, virus, fungi, plant or mammalian genome.

15. The nucleic acid sensor molecule of claim 1, wherein the effector component comprises a sequence derived from the Hepatitis C virus (HCV).

16. The method of claim 11, wherein the target nucleic acid comprises a sequence derived from the Hepatitis C virus (HCV) 5'-UTR.

20   17. The nucleic acid sensor molecule of claim 15, wherein the Hepatitis C virus (HCV) sequence is derived from the 5'-UTR.

18. The method of claim 16, wherein the Hepatitis C virus (HCV) sequence is dervied from the 5'-UTR.

19. A kit comprising the nucleic acid sensor molecule of claim 1.

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20. A nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components wherein, in response to an interaction of a single stranded RNA (ssRNA) having a single nucleotide polymorphism (SNP) with the nucleic acid sensor molecule in a system, the enzymatic nucleic acid component
5   catalyzes a chemical reaction resulting in a detectable response.

21. A nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components wherein, in response to an interaction of a single stranded DNA (ssDNA) having a single nucleotide polymorphism (SNP) with the nucleic acid sensor molecule in a system, the enzymatic nucleic acid component
10   catalyzes a chemical reaction resulting in a detectable response.

22. A nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components wherein, in response to an interaction of a single stranded RNA (ssRNA) with the nucleic acid sensor molecule in a system, the enzymatic nucleic acid component catalyzes a chemical reaction resulting in cleavage
15   of a predetermined nucleic acid molecule associated with a disease.

23. A nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components wherein, in response to an interaction of a single stranded DNA (ssDNA) with the nucleic acid sensor molecule in a system, the enzymatic nucleic acid component catalyzes a chemical reaction resulting in cleavage
20   of a predetermined nucleic acid molecule associated with a disease.

24. A nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components wherein, in response to an interaction of a single stranded RNA (ssRNA) having a single nucleotide polymorphism (SNP) with the nucleic acid sensor molecule in a system, the enzymatic nucleic acid component
25   catalyzes a chemical reaction resulting in ligation of a predetermined nucleic acid molecule to another predetermined nucleic acid molecule.

25. A nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components wherein, in response to an interaction of a single

stranded DNA (ssDNA) having a single nucleotide polymorphism (SNP) with the nucleic acid sensor molecule in a system, the enzymatic nucleic acid component catalyzes a chemical reaction resulting in ligation of a predetermined RNA molecule to another predetermined RNA molecule.

5. 26. A method comprising:

   a. contacting the nucleic acid sensor molecule of claim 1 with a system comprising at least one ssRNA having a single nucleotide polymorphism (SNP) under conditions suitable for the enzymatic nucleic acid component of the nucleic acid sensor molecule to catalyze a chemical reaction

10   resulting in a detectable response; and

   b. assaying for said chemical reaction.

   27. A method comprising:

   a. contacting the nucleic acid sensor molecule of claim 2 with a system comprising at least one ssDNA having a single nucleotide polymorphism

15   (SNP) under conditions suitable for the enzymatic nucleic acid component of the nucleic acid sensor molecule to catalyze a chemical reaction resulting in a detectable response; and

   b. assaying for said chemical reaction resulting in a detectable response.

   28. The nucleic acid sensor molecule of claim 20 or claim 21, wherein said chemical

20   reaction is cleavage of a phosphodiester internucleotide linkage.

   29. The nucleic acid sensor molecule of claim 20 or claim 21, wherein said chemical reaction is ligation of a predetermined nucleic acid molecule to the nucleic acid sensor molecule.

   30. The nucleic acid sensor molecule of claim 20 or claim 21, wherein said chemical

25   reaction is ligation of a predetermined nucleic acid molecule to another predetermined nucleic acid molecule.

Figure 1: Half-Zinzyme Construct

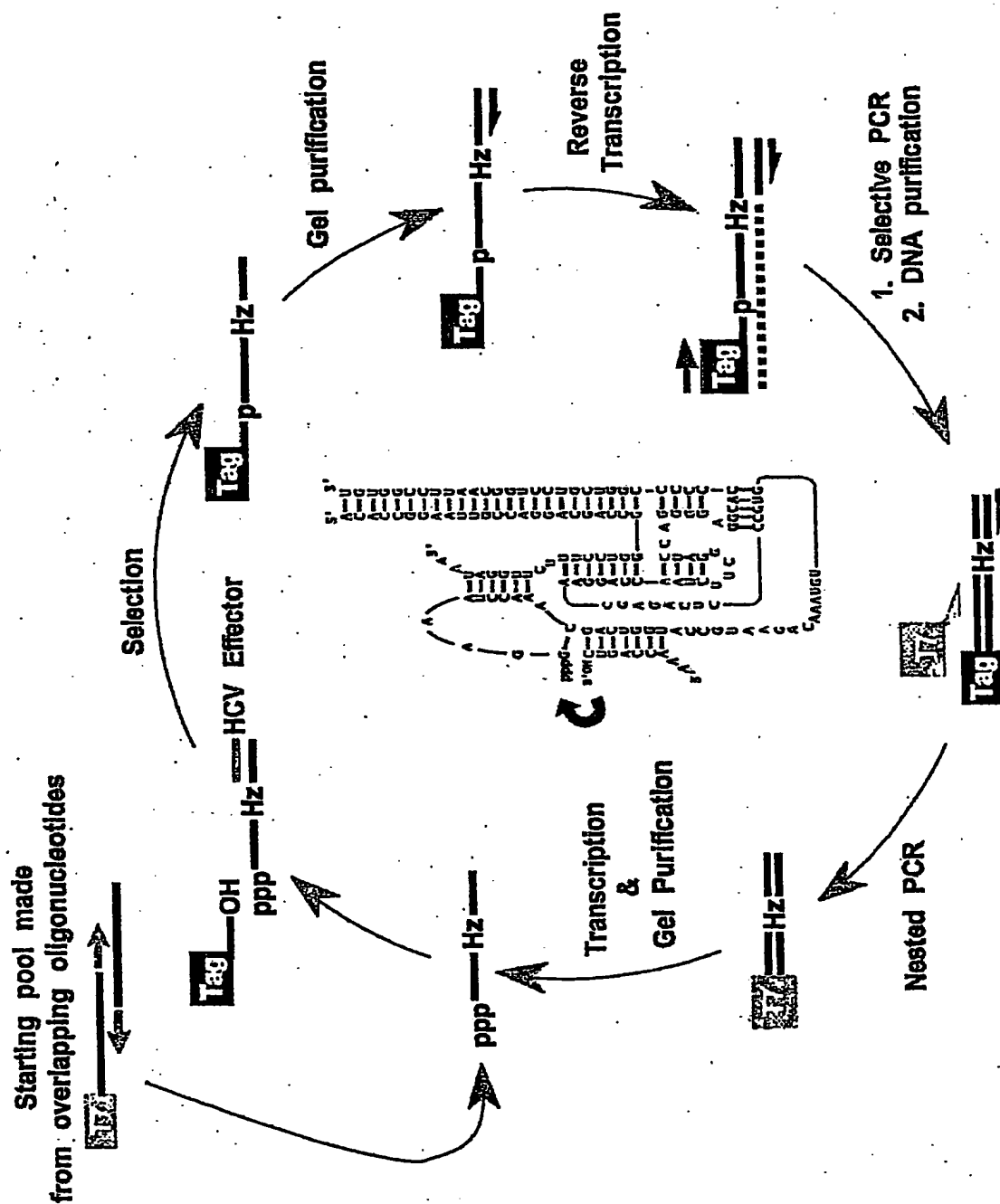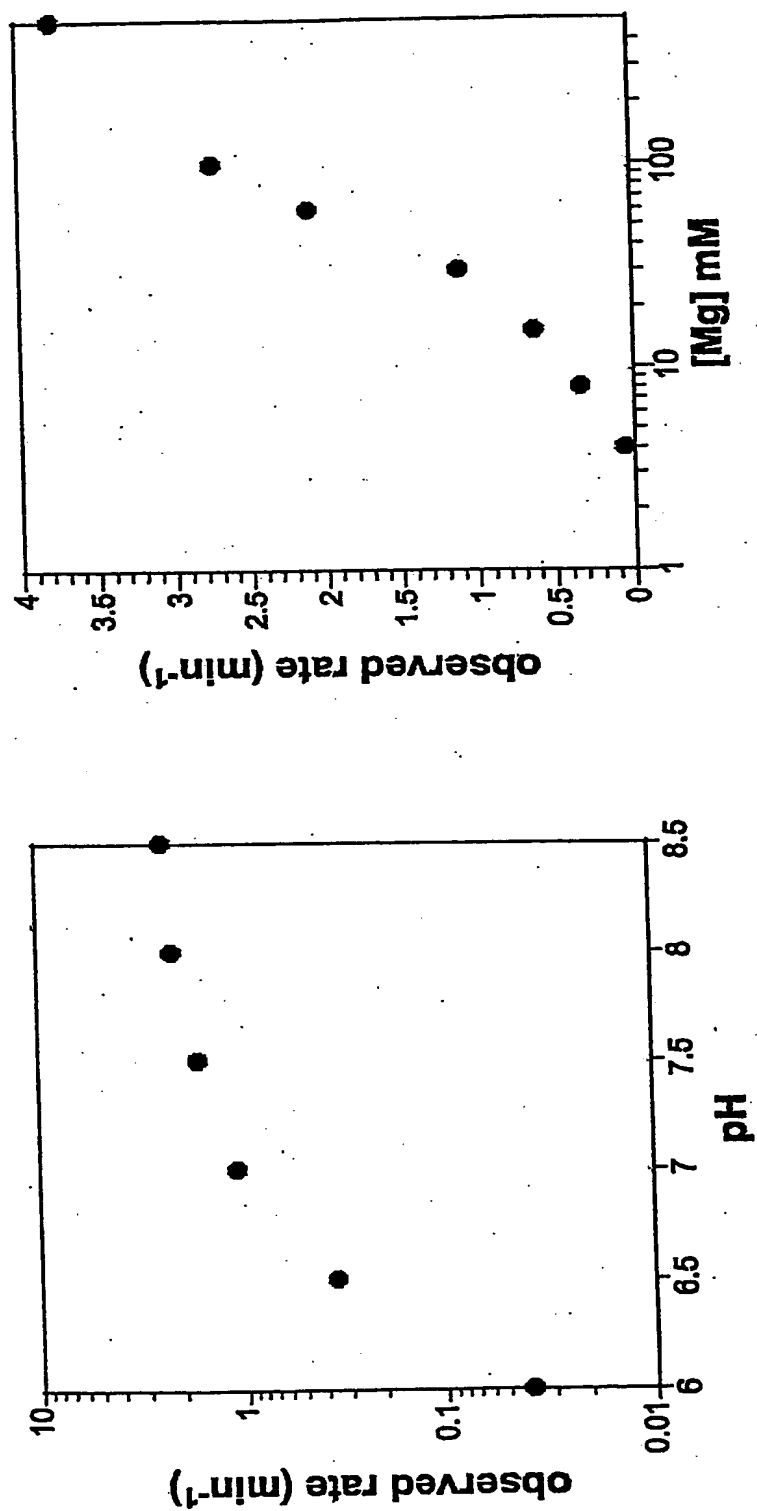Figure 2

a

b

Figure 3

Figure 4

Figure 5: Halfzyme Ligase
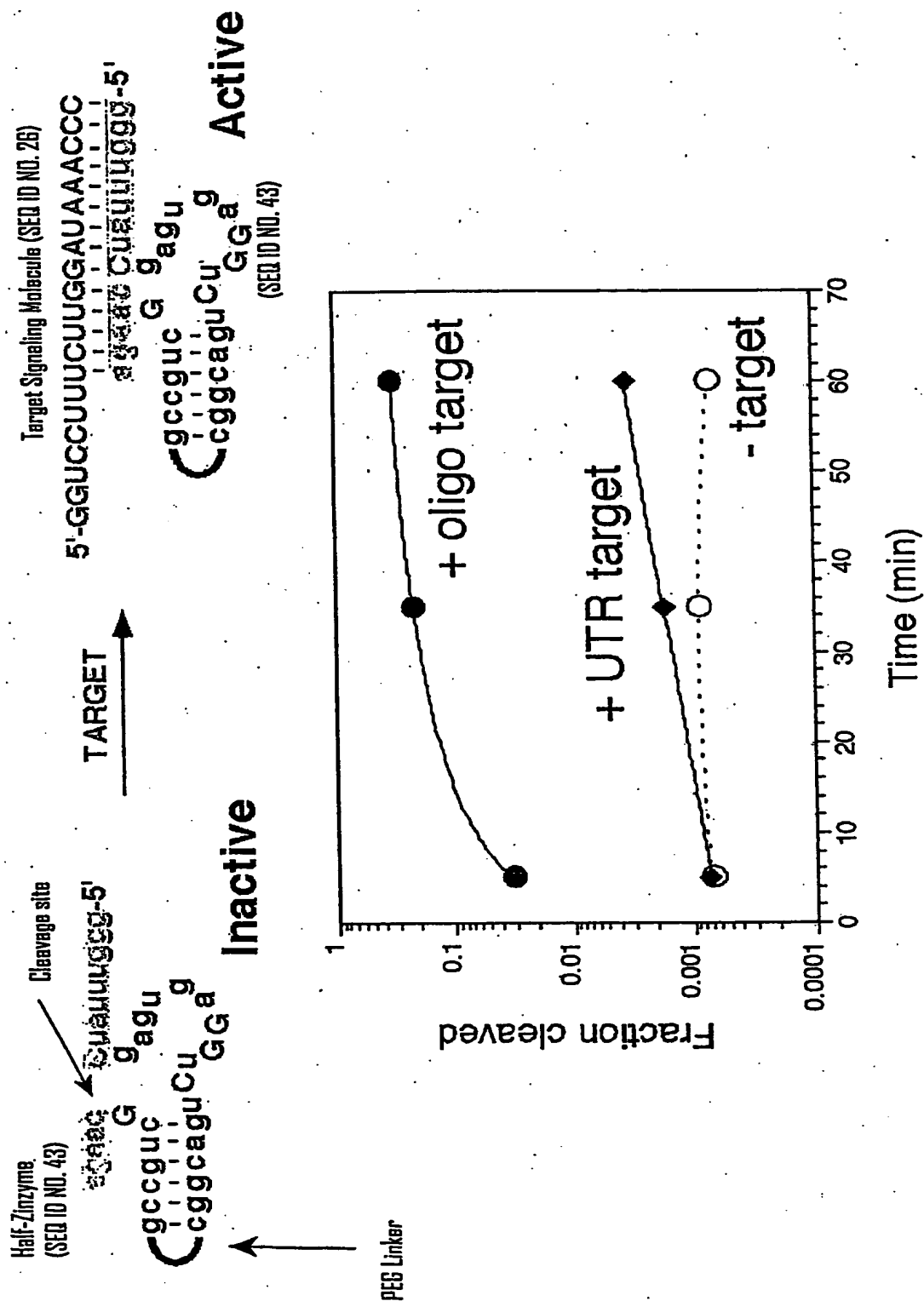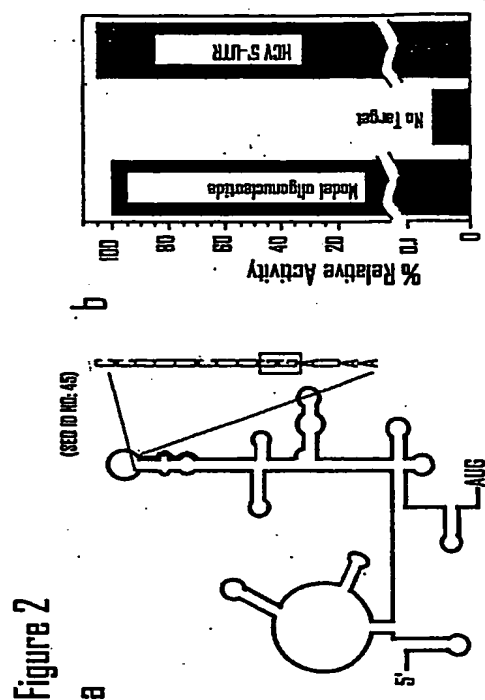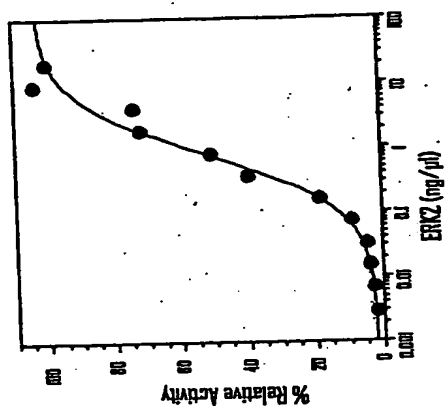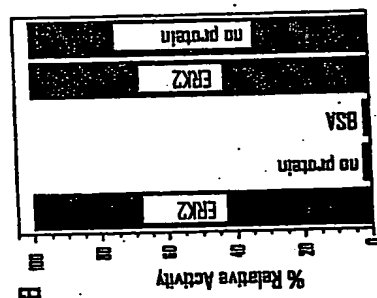
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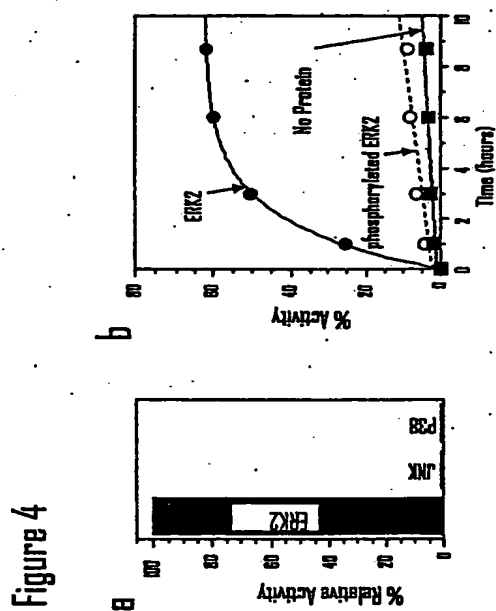
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Figure 6: Secondary structure of HCV 5'-UTR

(SEQ ID NO 48)

Figure 7

Design of SNP Detection using Halfzyme-AZB7.1
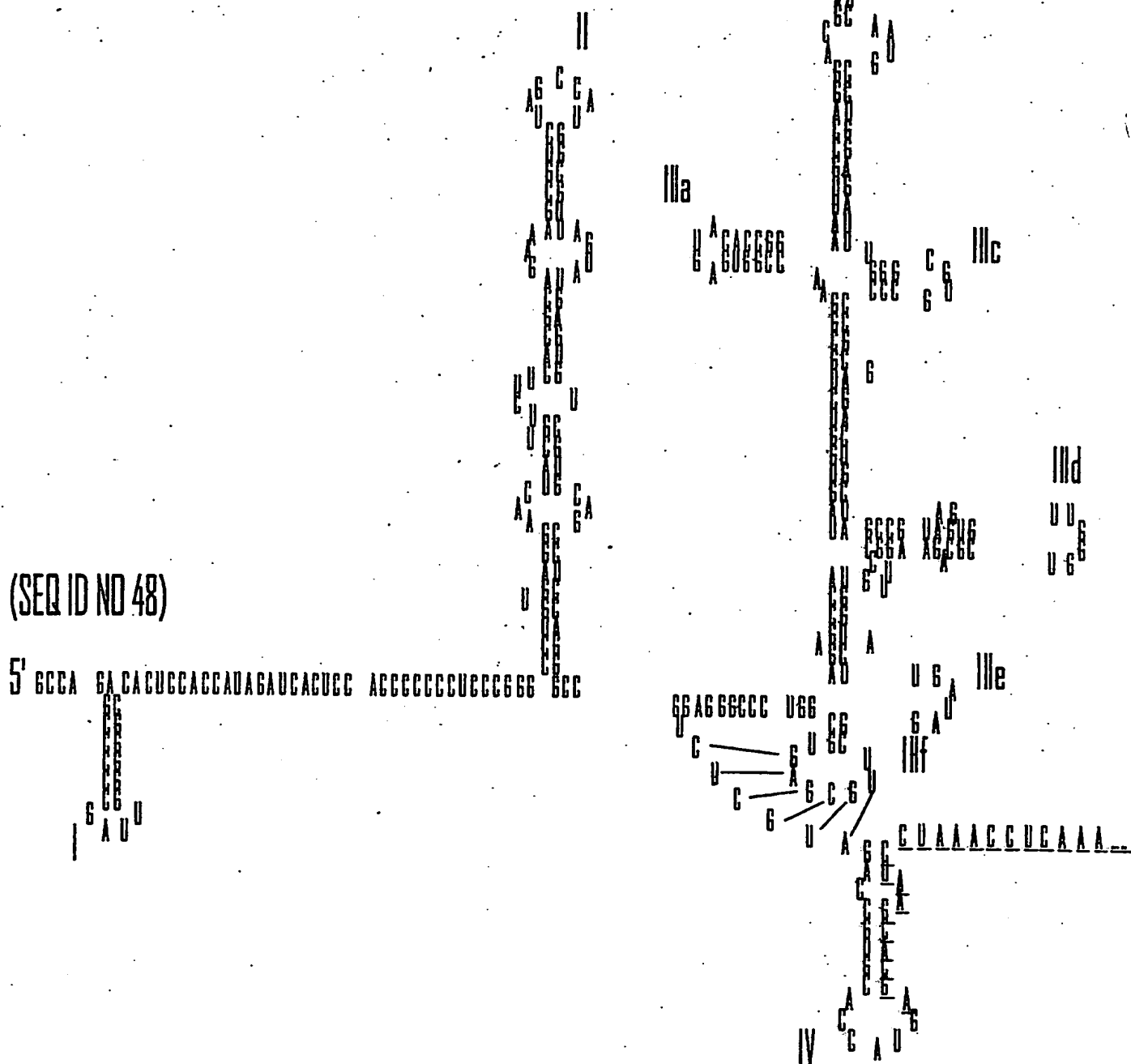
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```
                aG G
             g u  Cugacggc
          g u g a g       cugccg
AZB7.1             G   c g a c g g g  -3'

        5'-a g c g c  C
```

		SEQ ID NO:
Target HBV 1887(True)=AZB7-GG 3'-T C G C G – G C T G C C C-5' (SNPT-1)		50
AZB7-AG 3'-T C G C A – G C T G C C C-5' (SNPT-2)		51
AZB7-TG 3'-T C G C T – G C T G C C C-5' (SNPT-3)		52
AZB7-CG 3'-T C G C C – G C T G C C C-5' (SNPT-4)		53
AZB7-GA 3'-T C G C G – A C T G C C C-5' (SNPT-5)		54
AZB7-GT 3'-T C G C G – T C T G C C C-5' (SNPT-6)		55
AZB7-GC 3'-T C G C G – C C T G C C C-5' (SNPT-7)		56
		57

RNA HBV 1433   <u>3'-U C G C G – G C U G C C C-5'</u>   58

Figure 8: Single Nucleotide Polymorphism (SNP) Detection

Figure 9A

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(SEQ ID NO: 62)
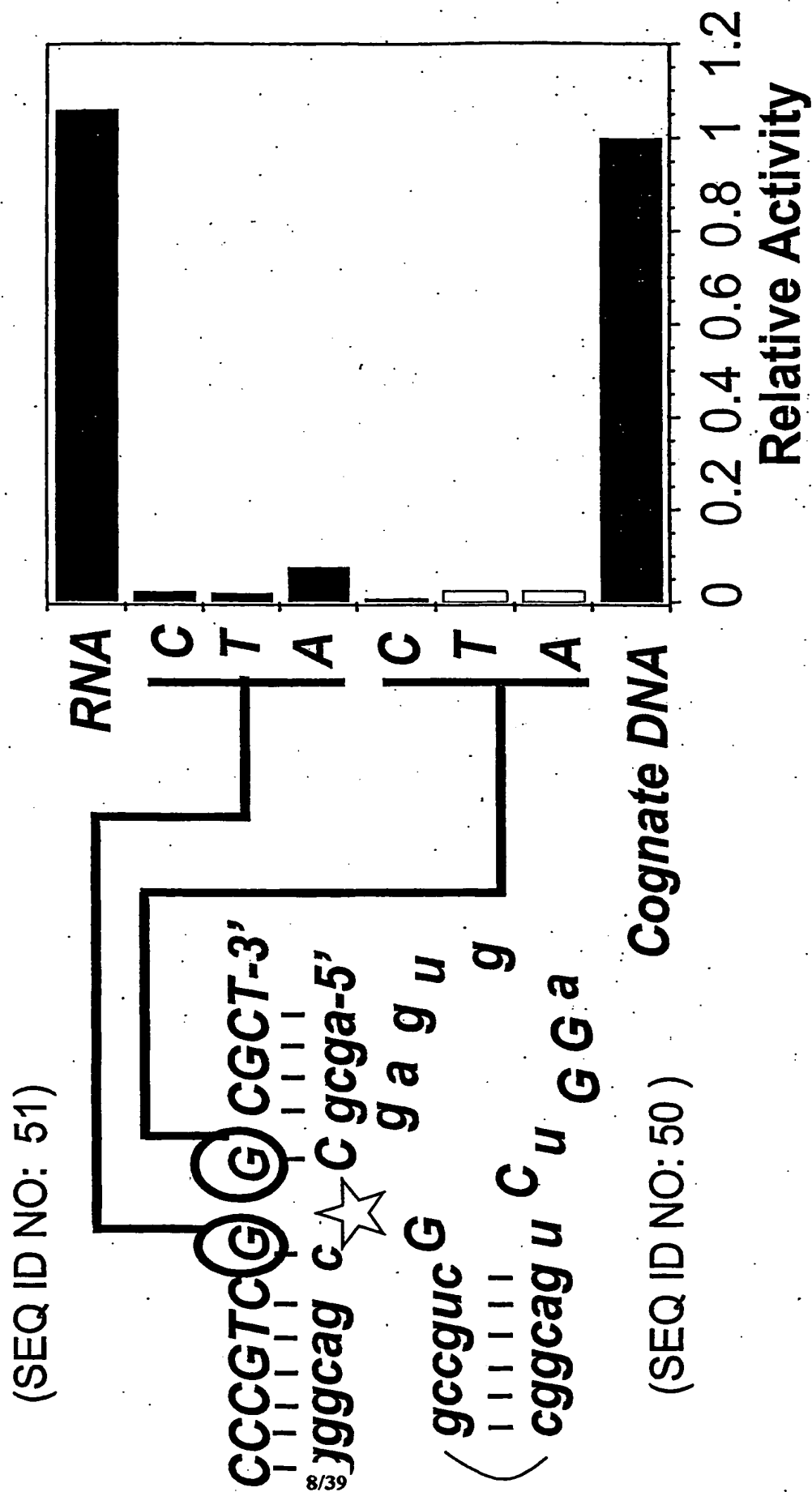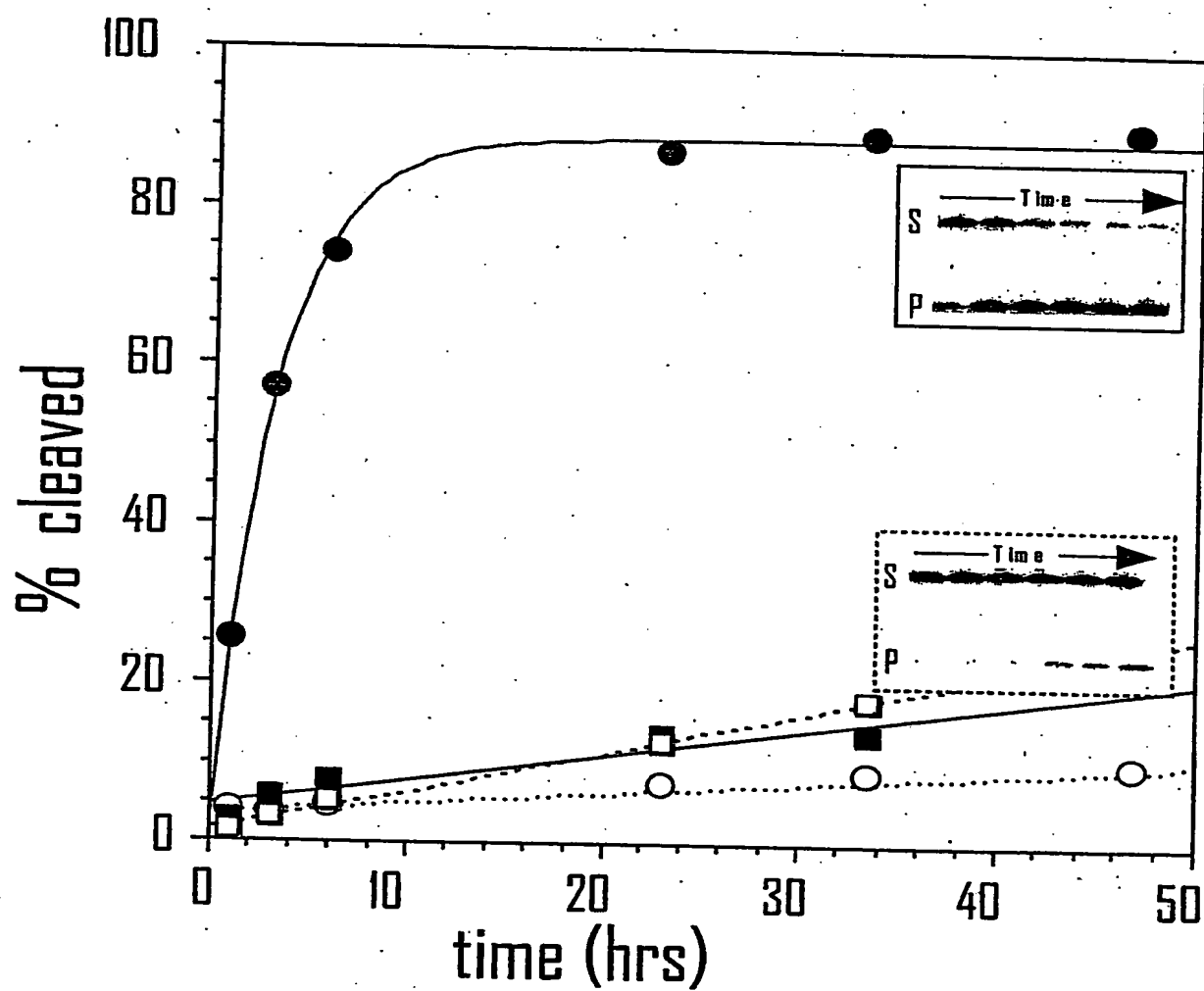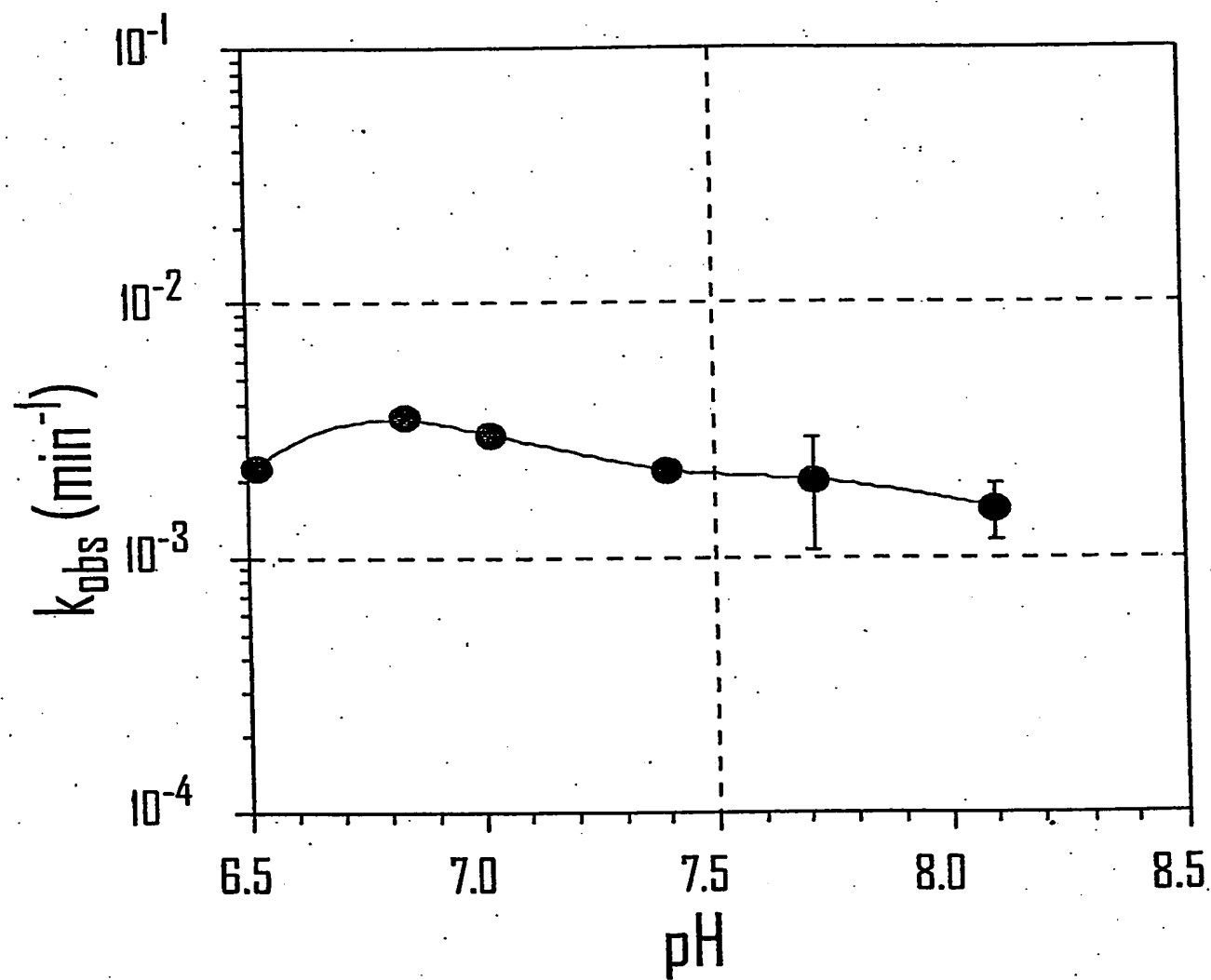
Figure 9B

Figure 9C

Figure 9D
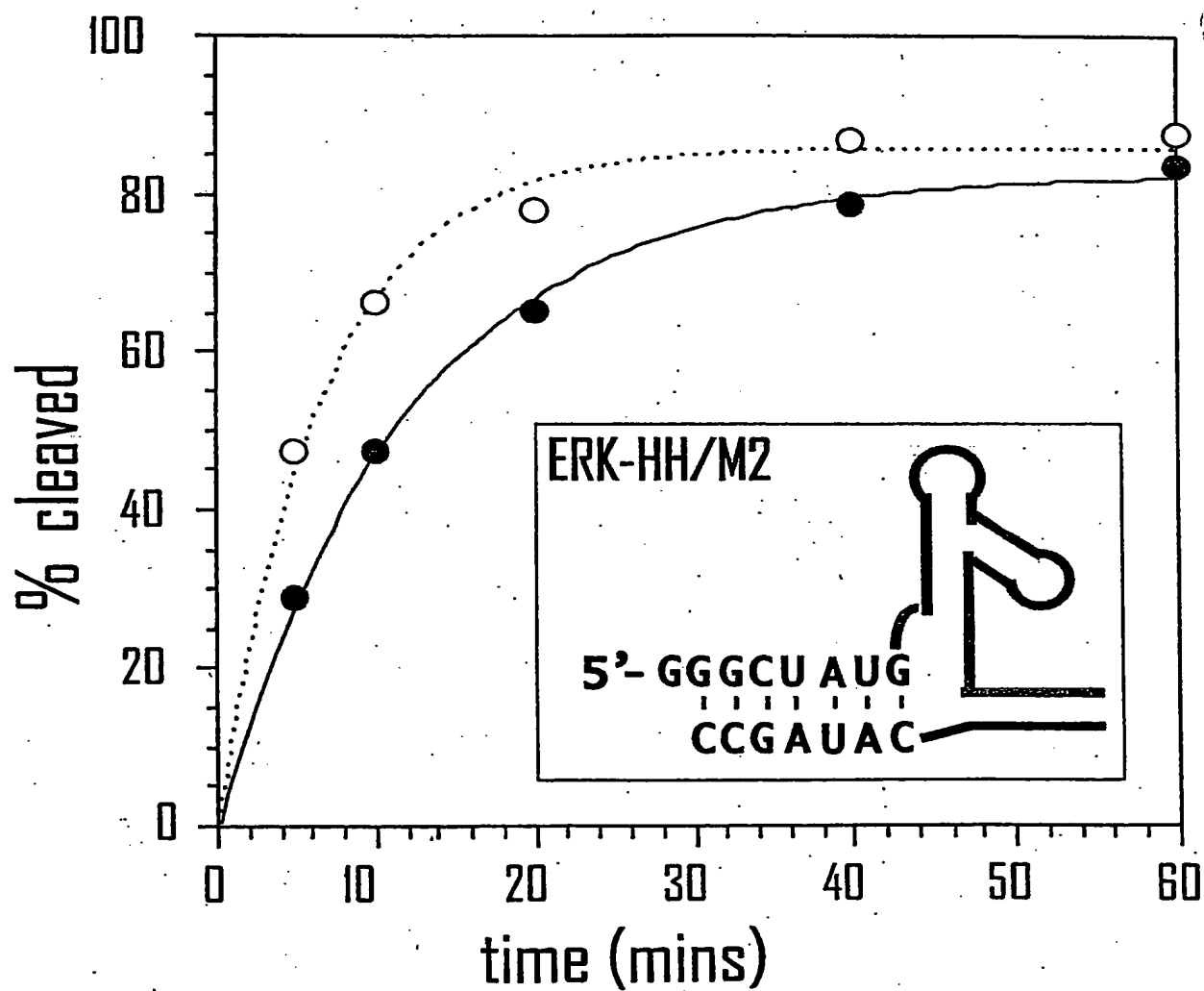
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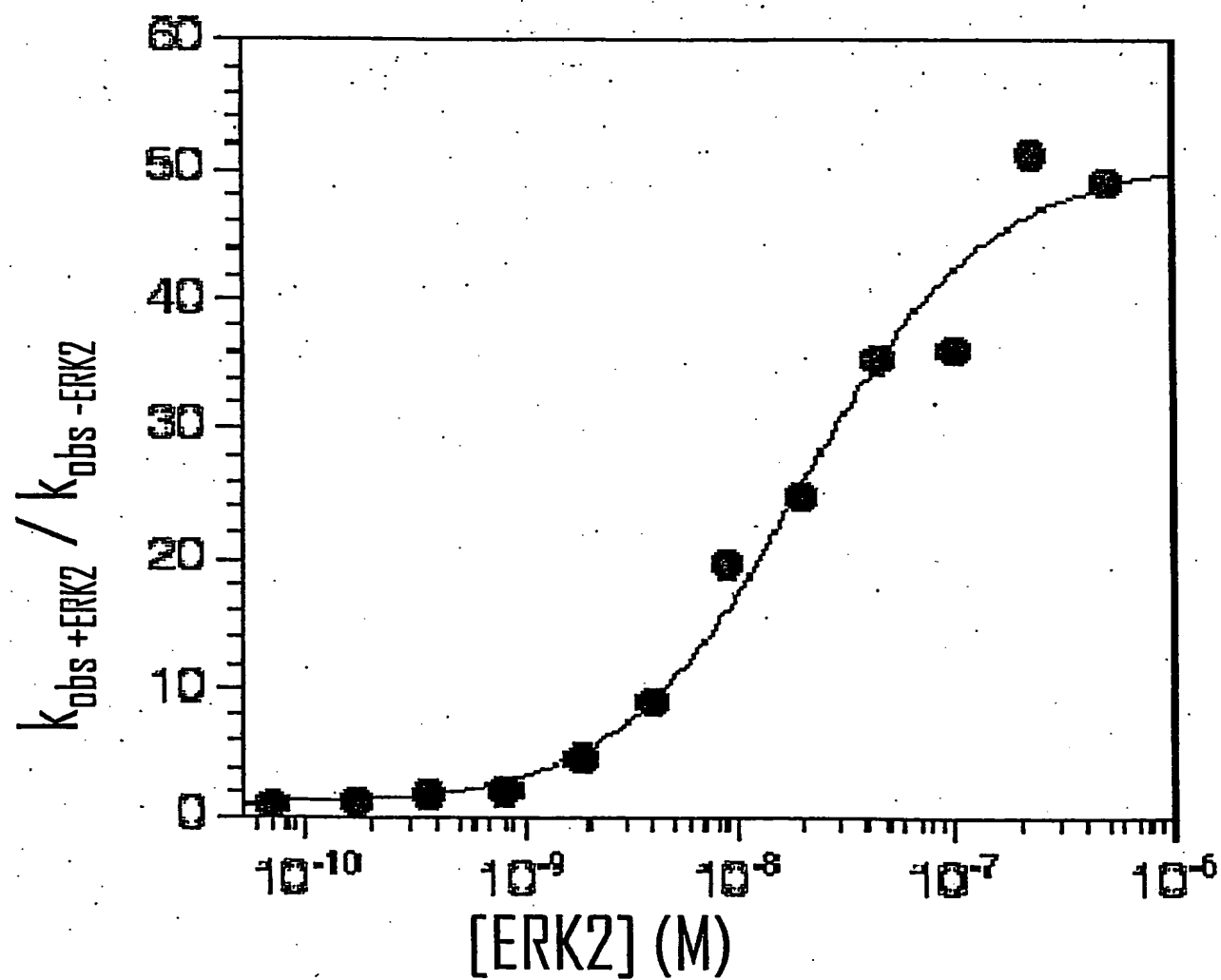
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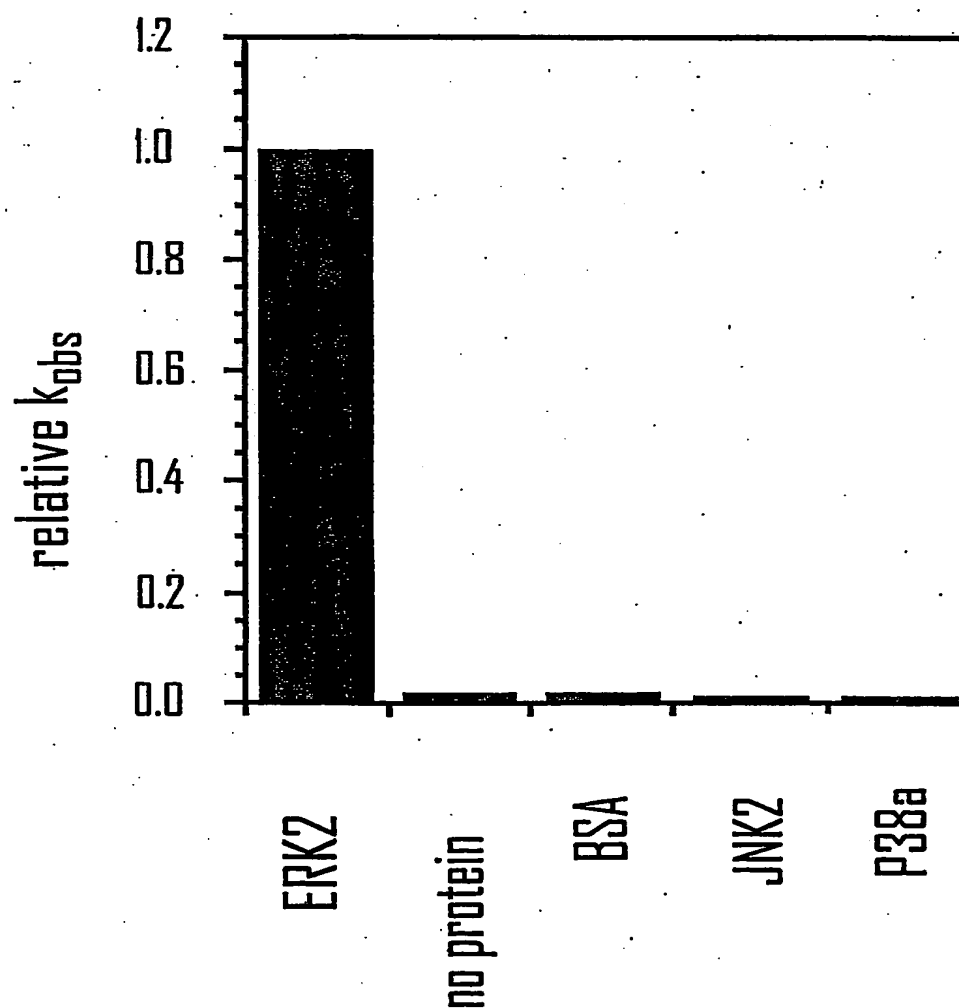
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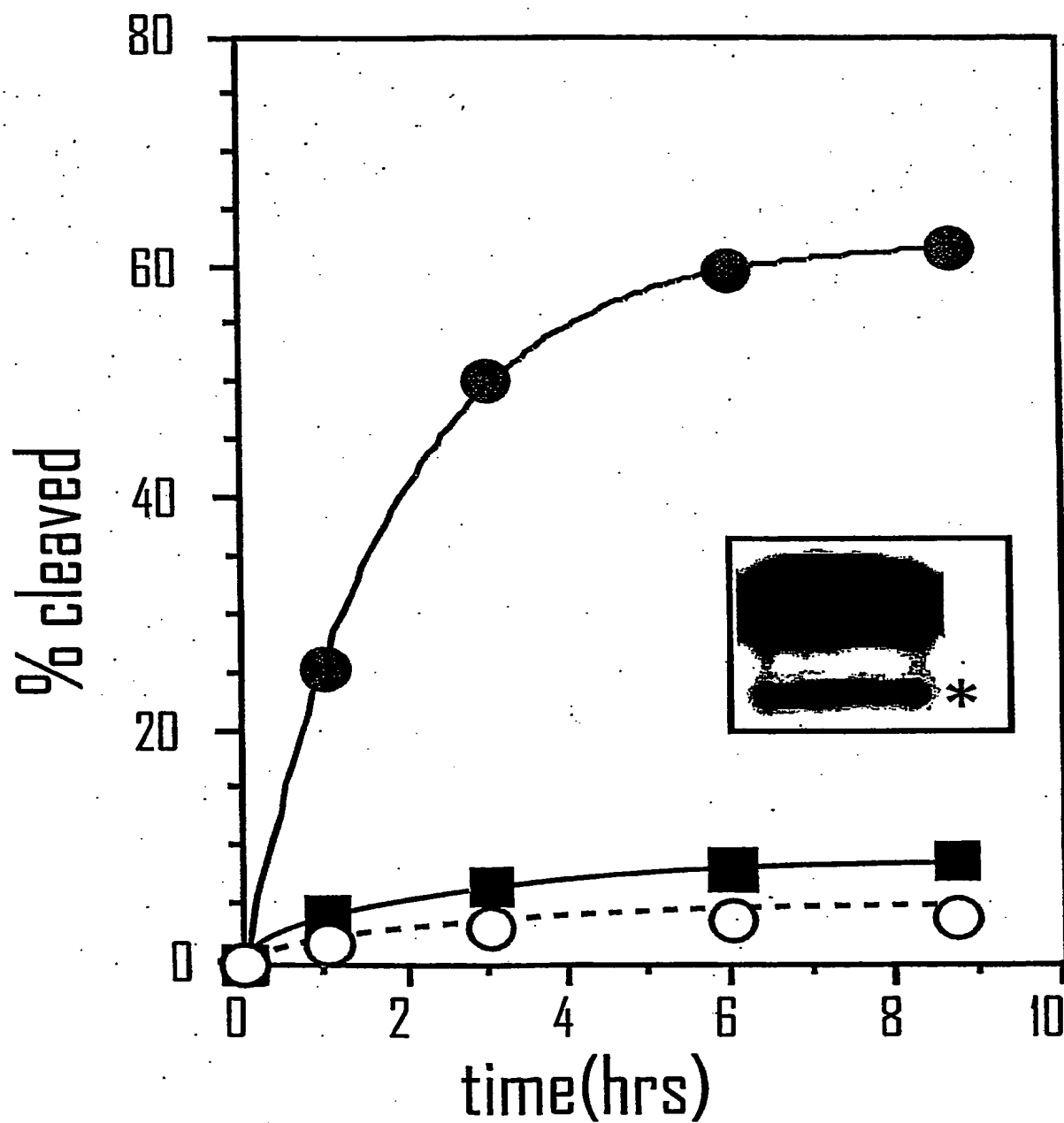
Figure 12A

Figure 12B

Figure 13A

ERK2-HH

Constitutive Enzymatic Nucleic Acid

Figure 13B

Figure 14A

(SEQ ID NO: 63)
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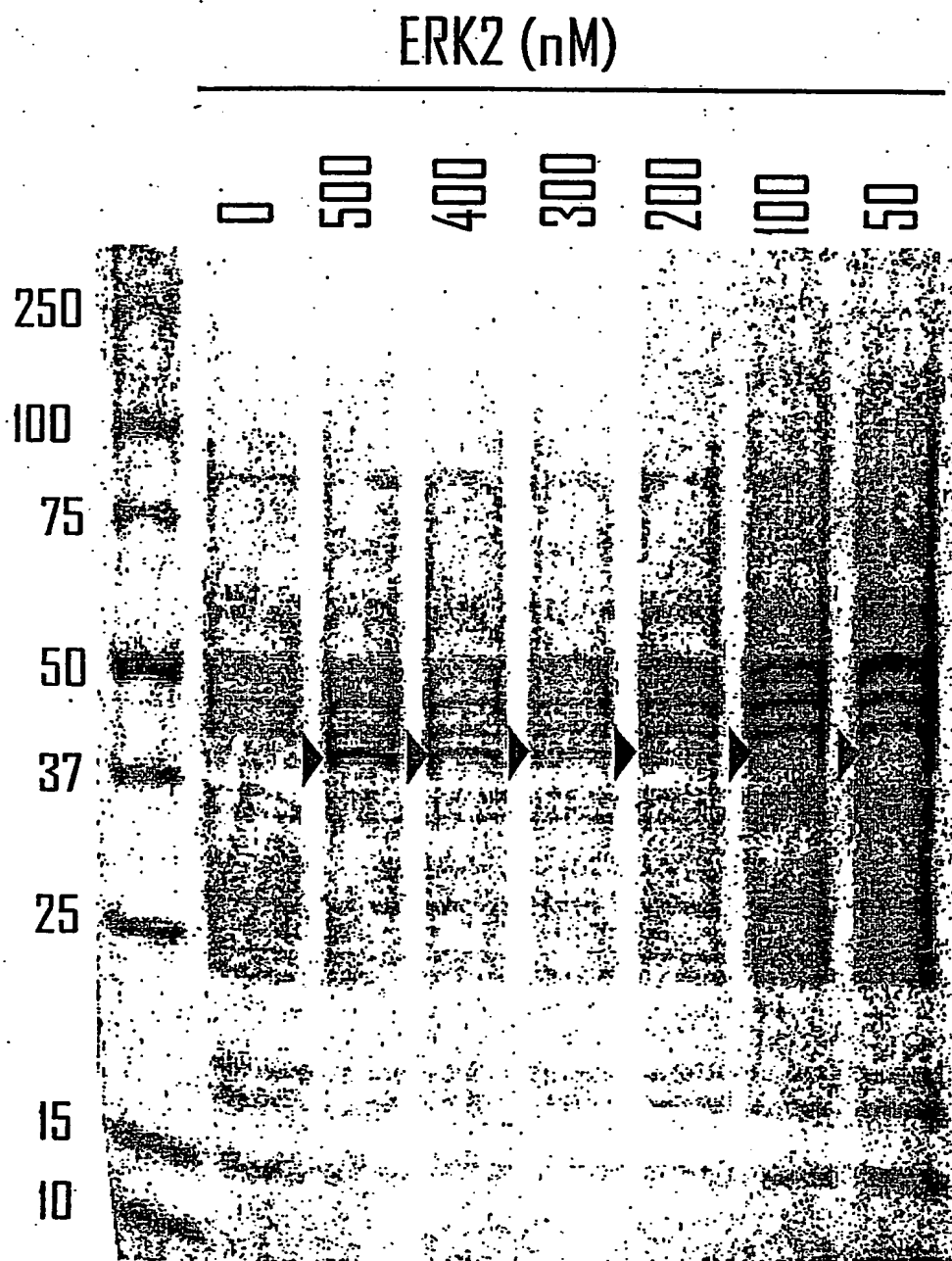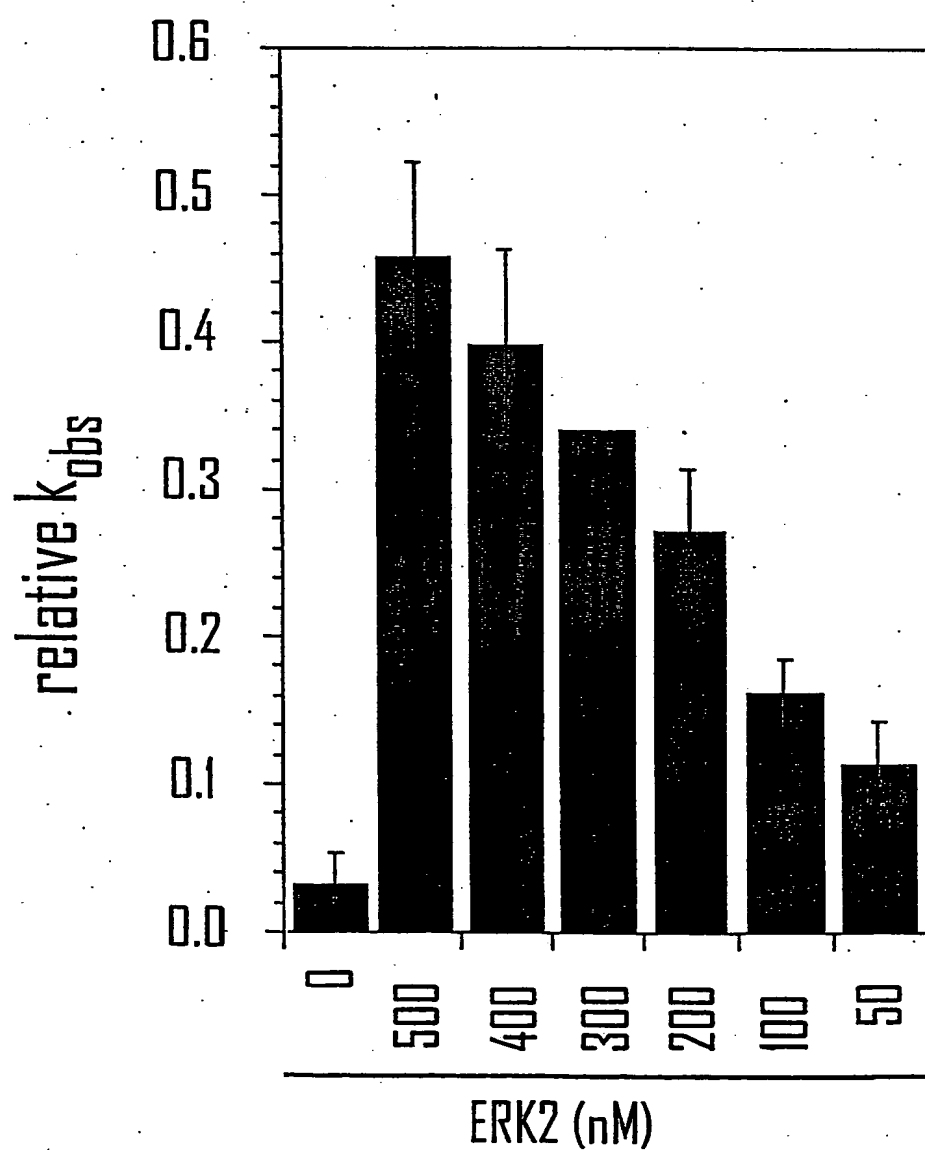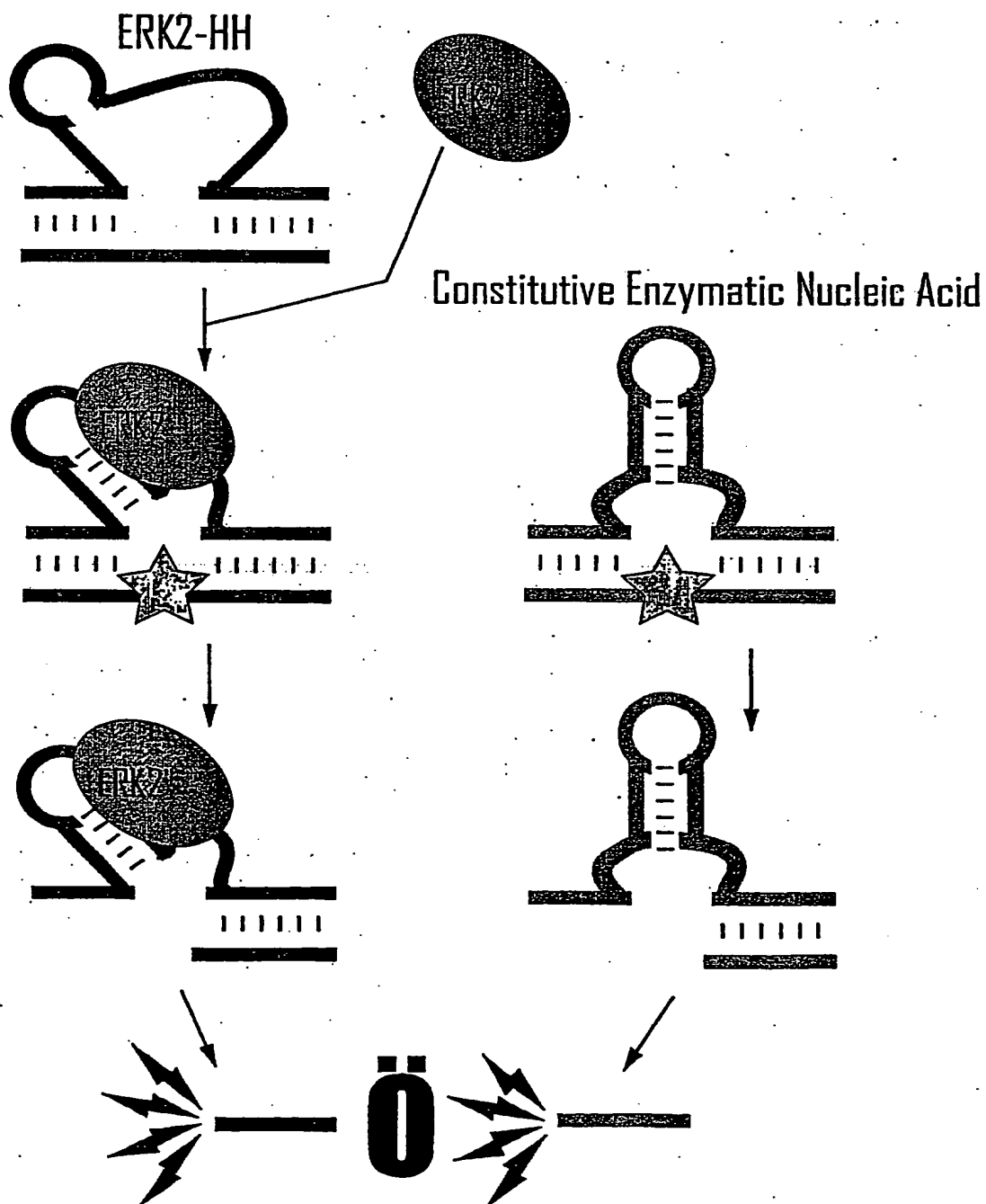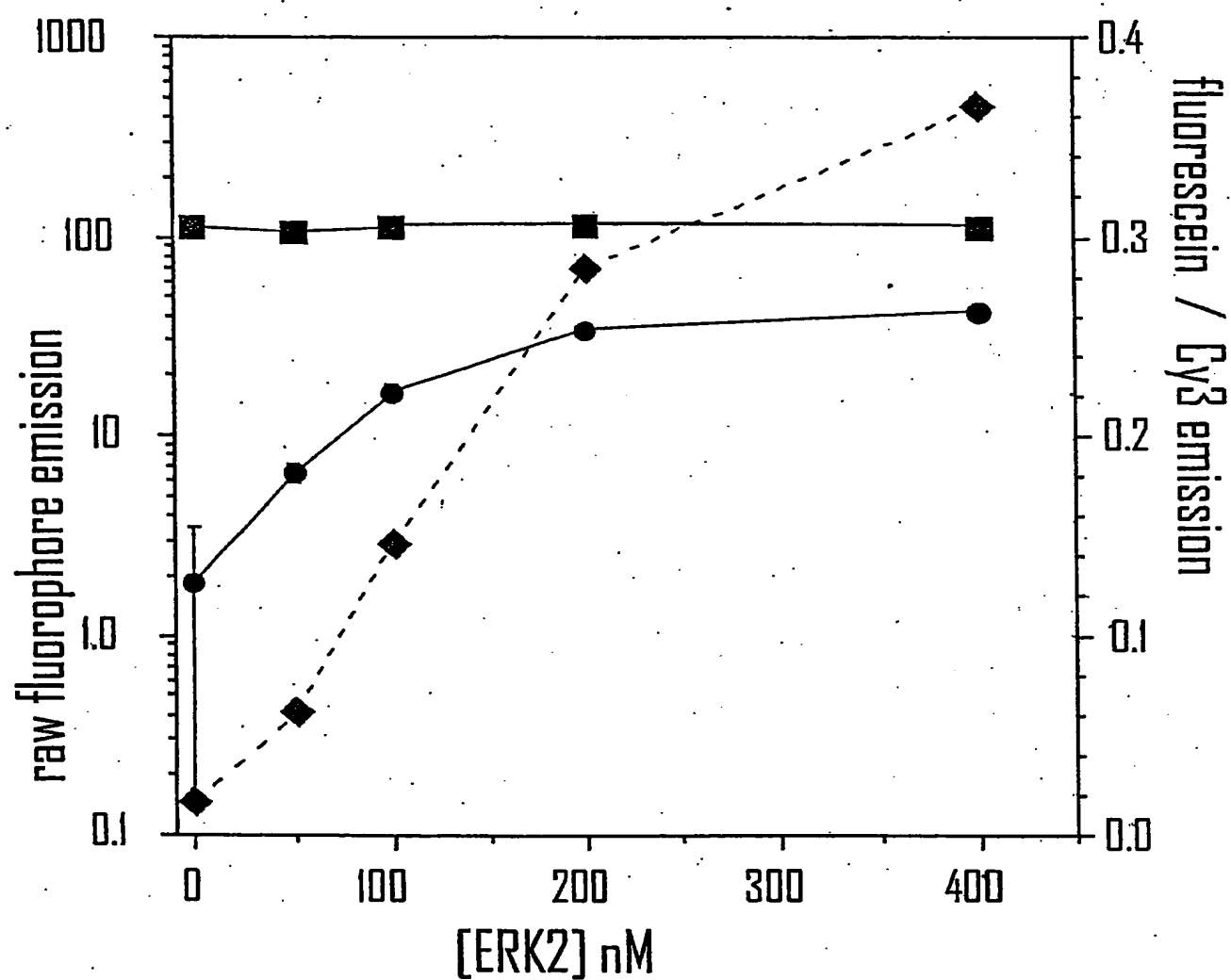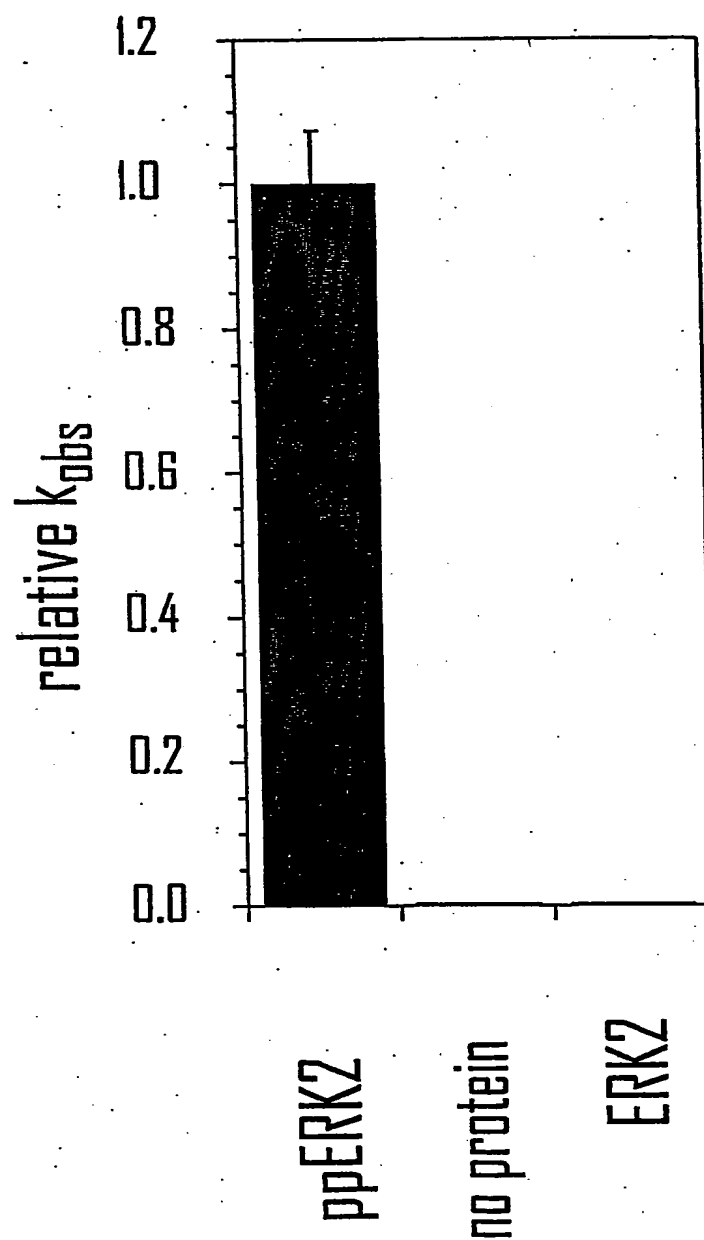
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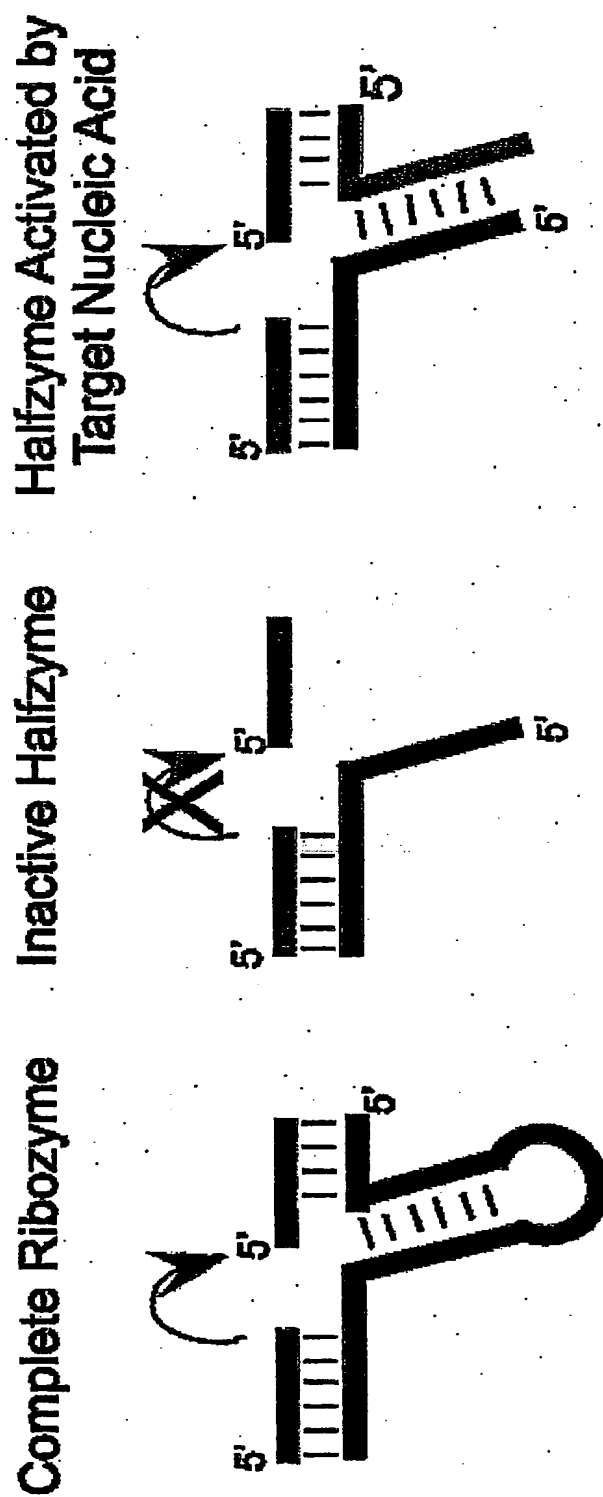
Figure 15

Figure 16
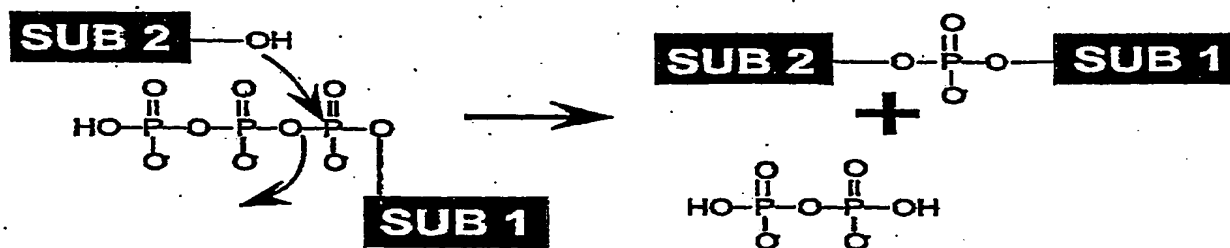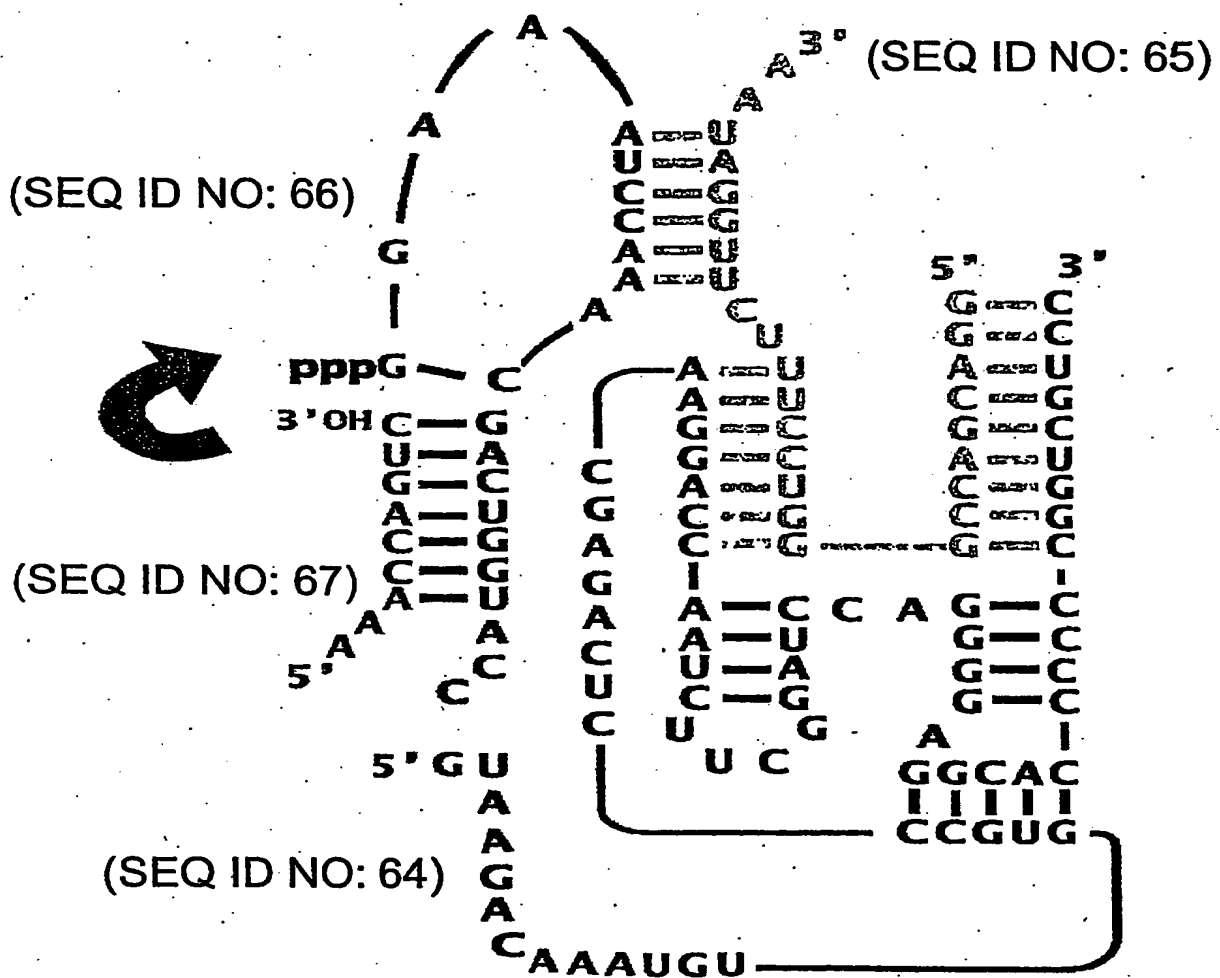
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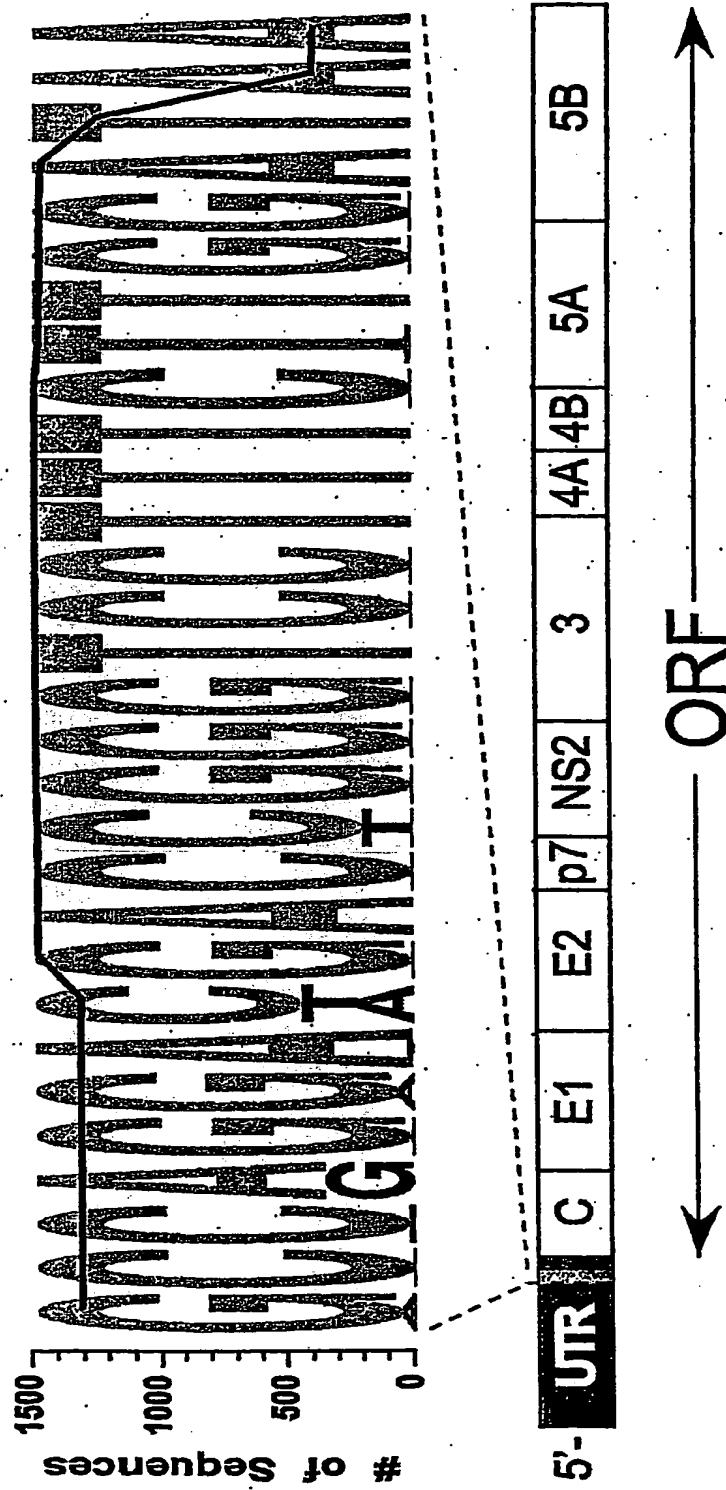
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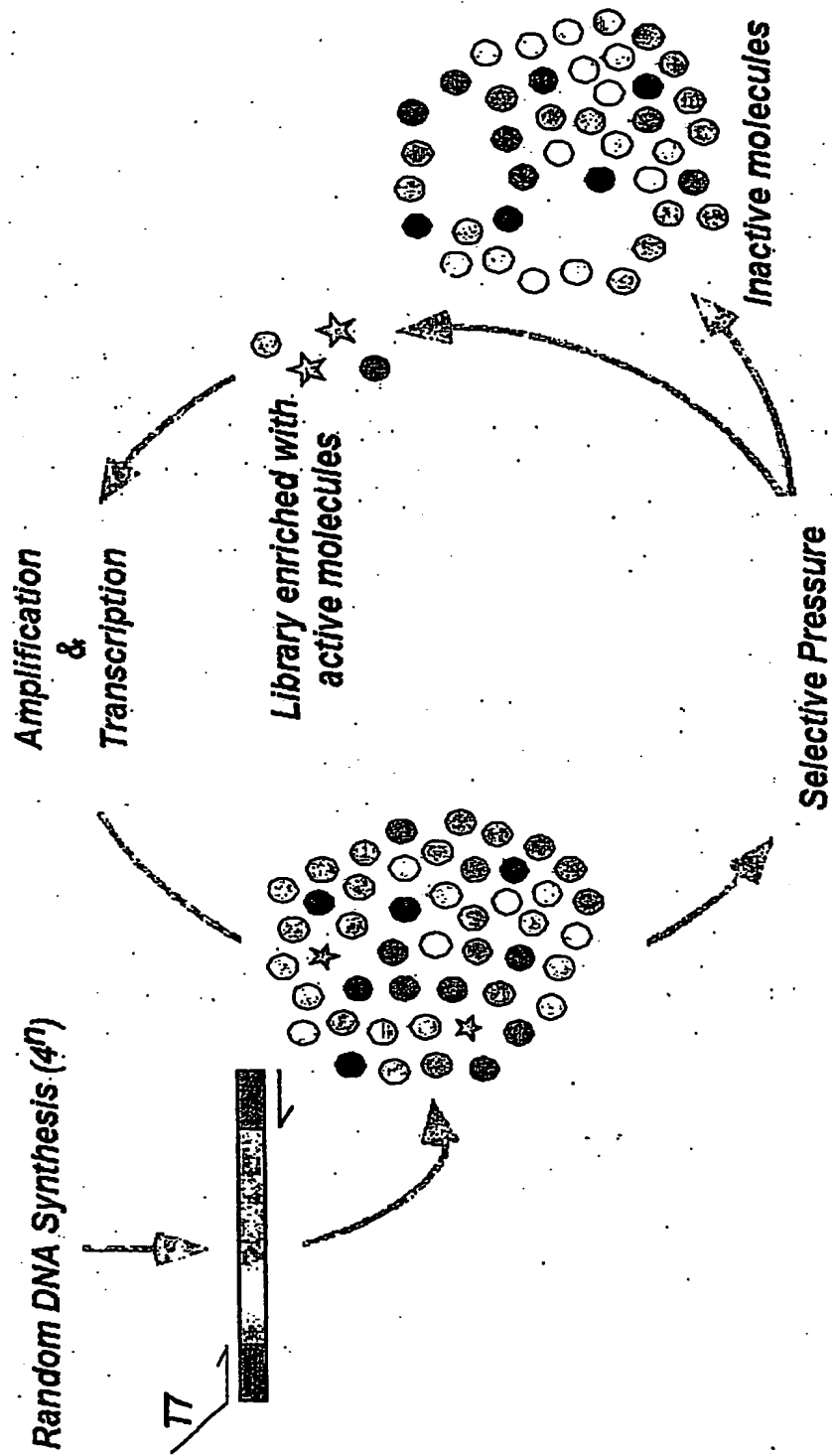
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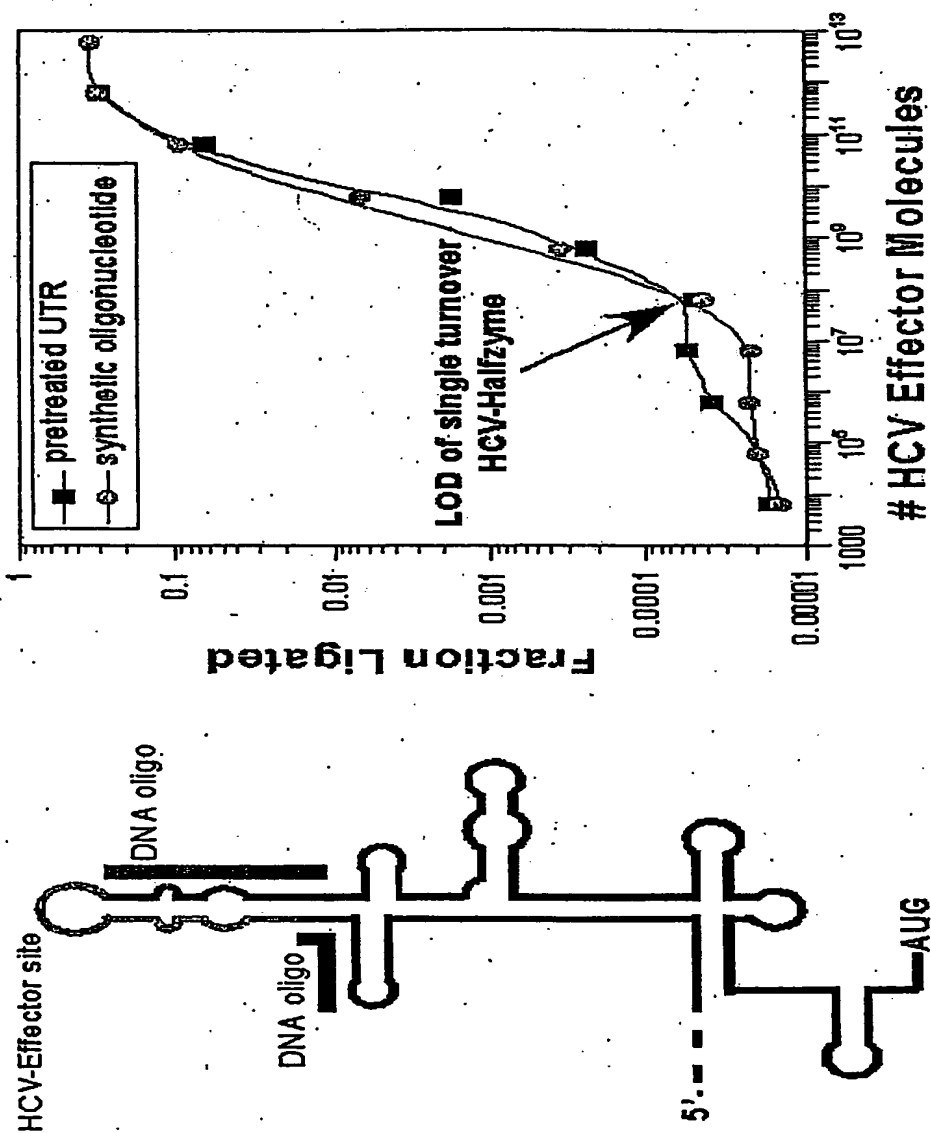
Figure 20

Figure 21

Figure 22

Figure 23
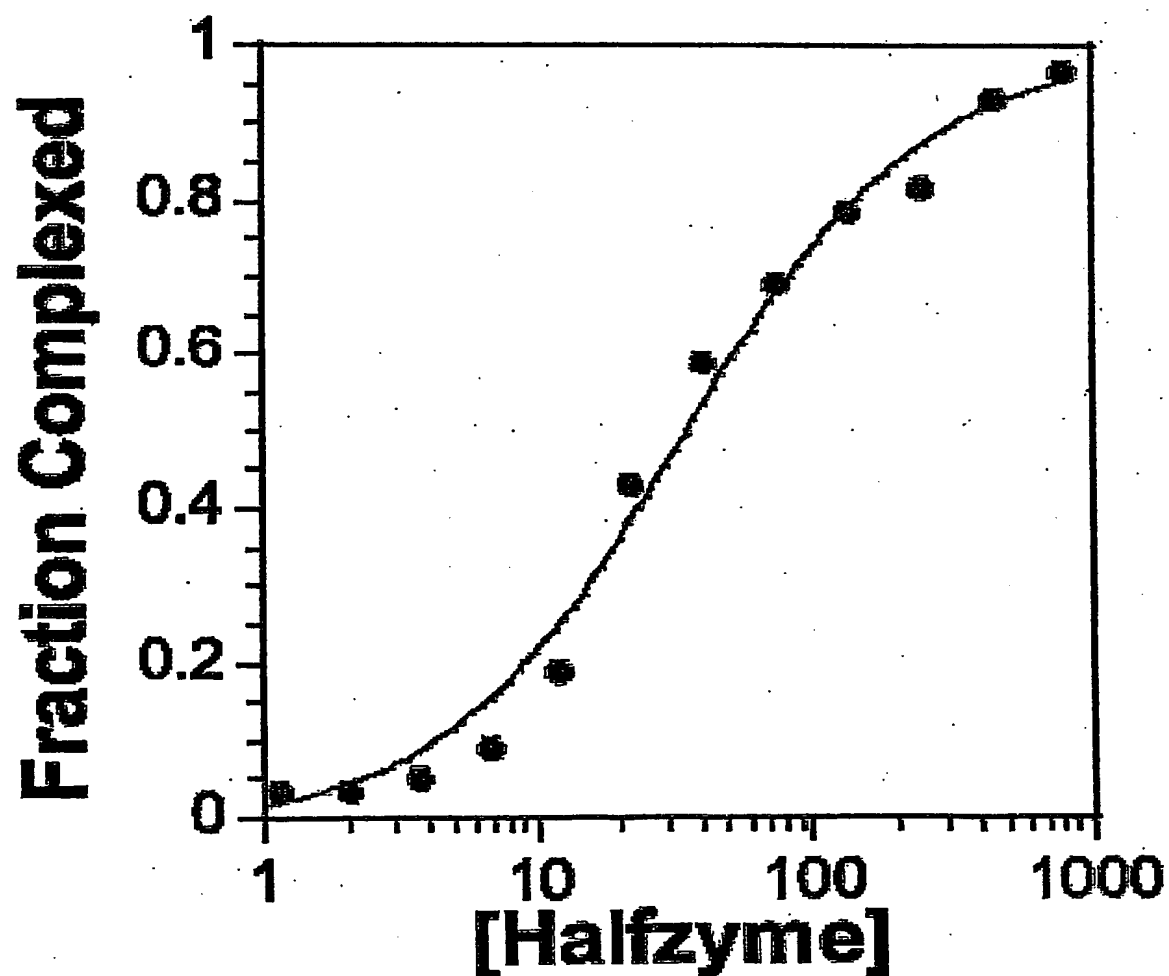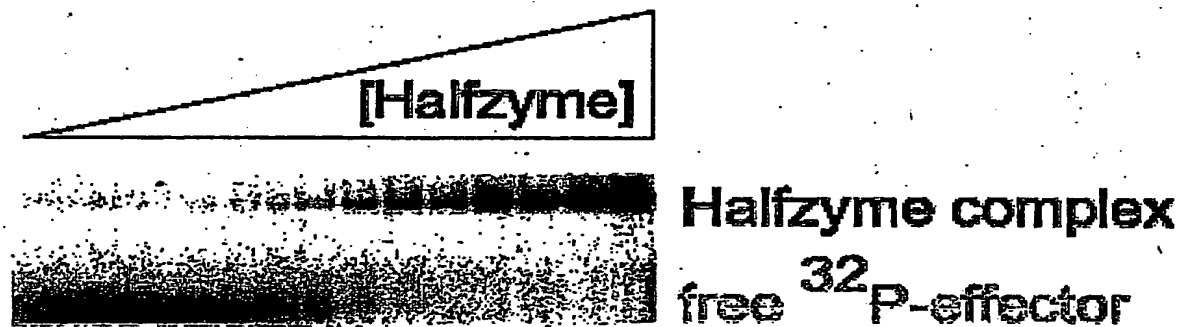
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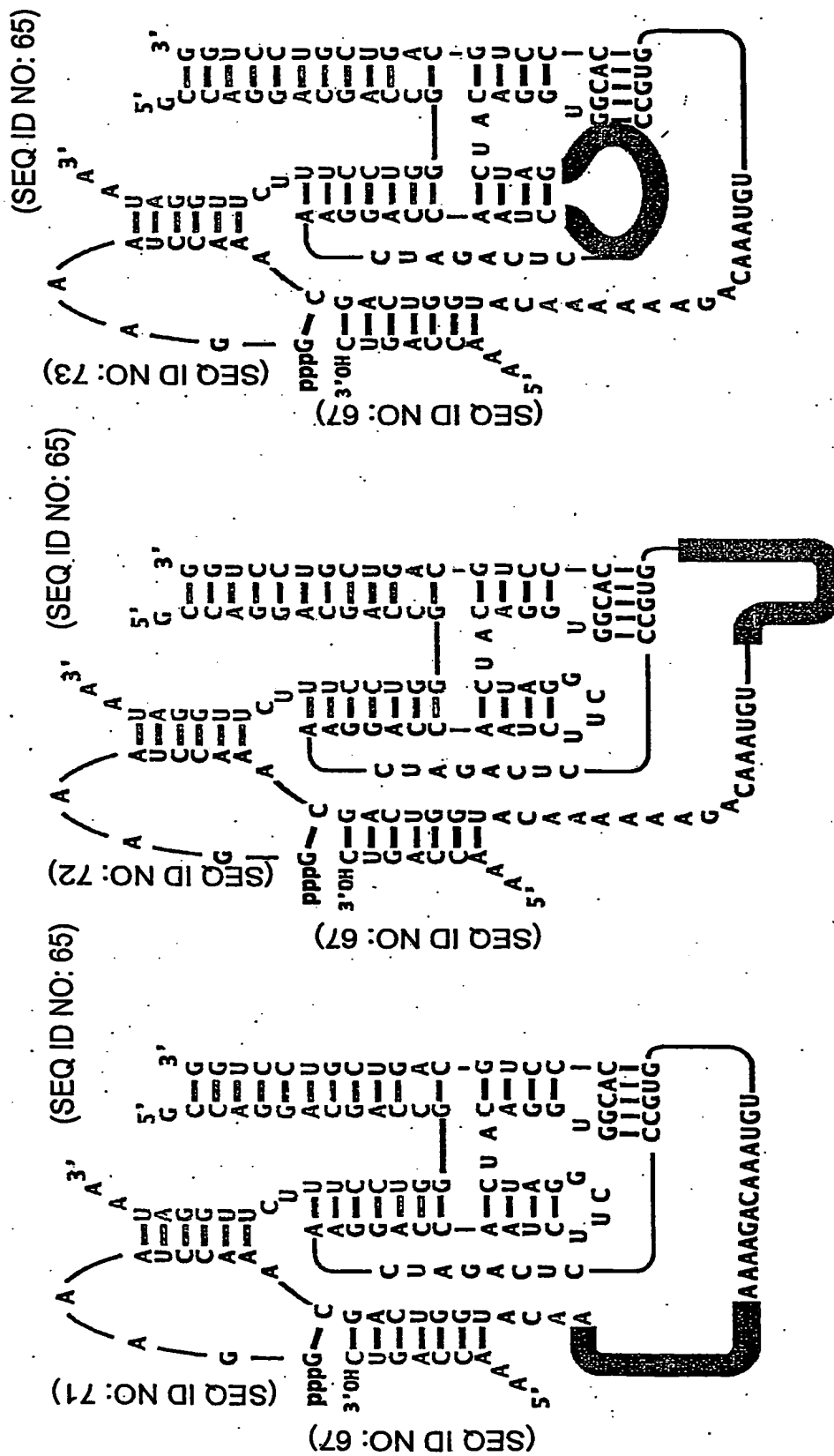
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Figure 26

Figure 27

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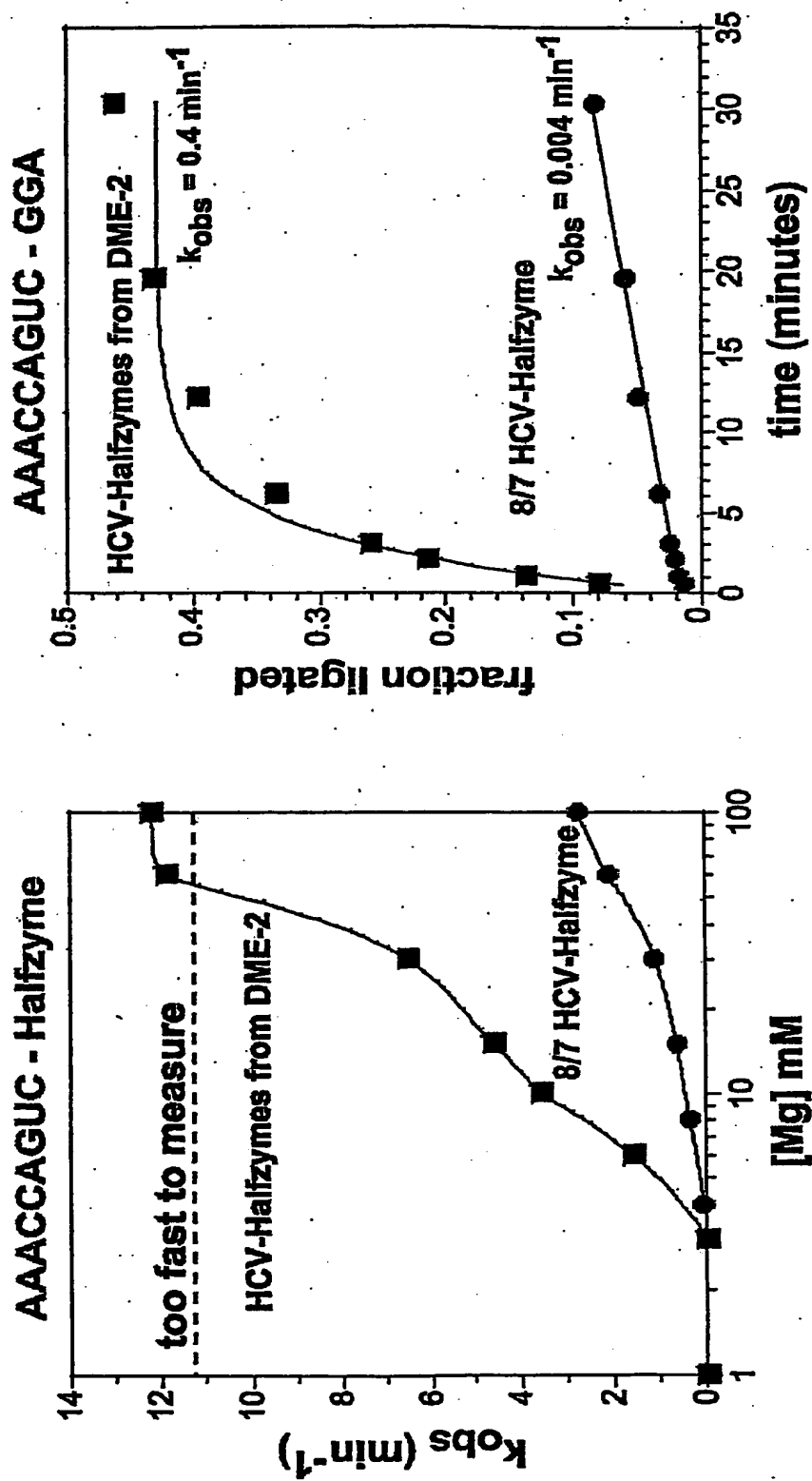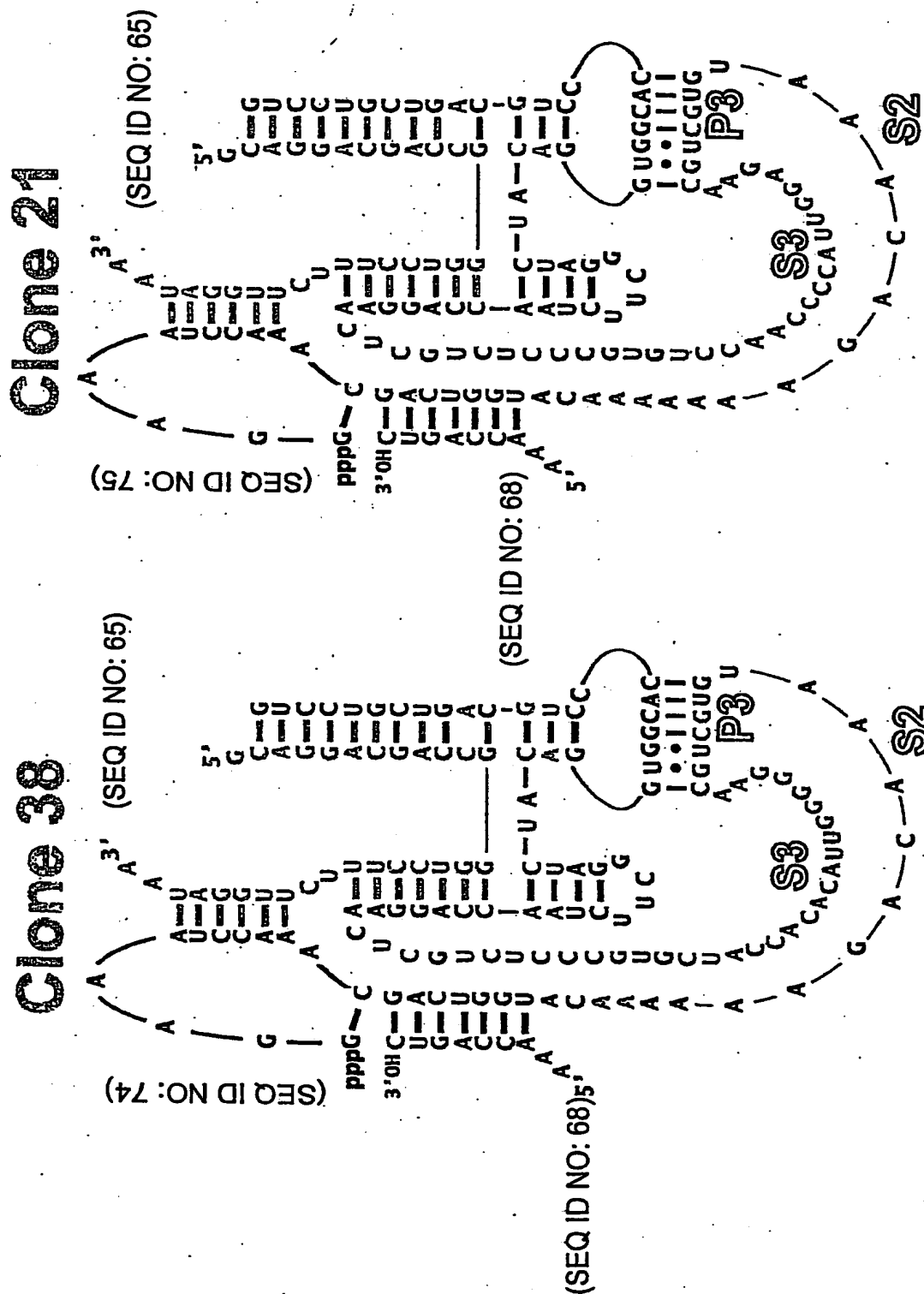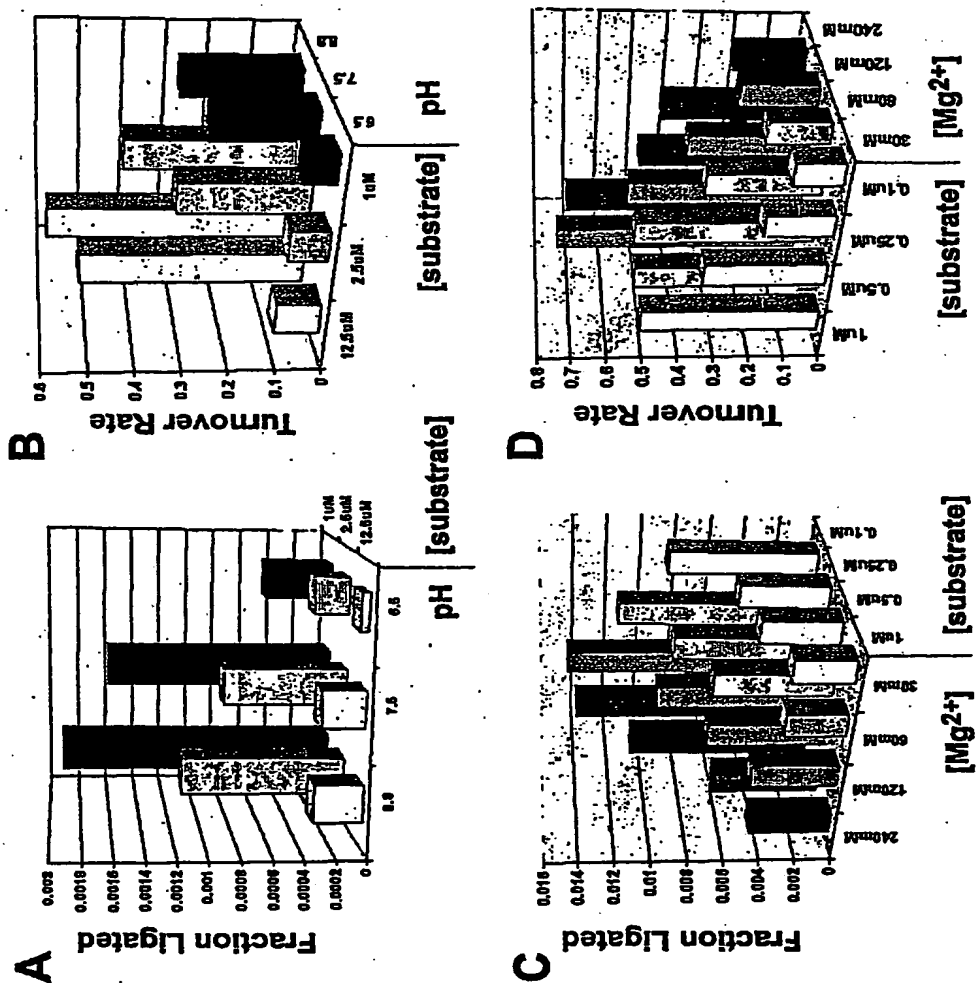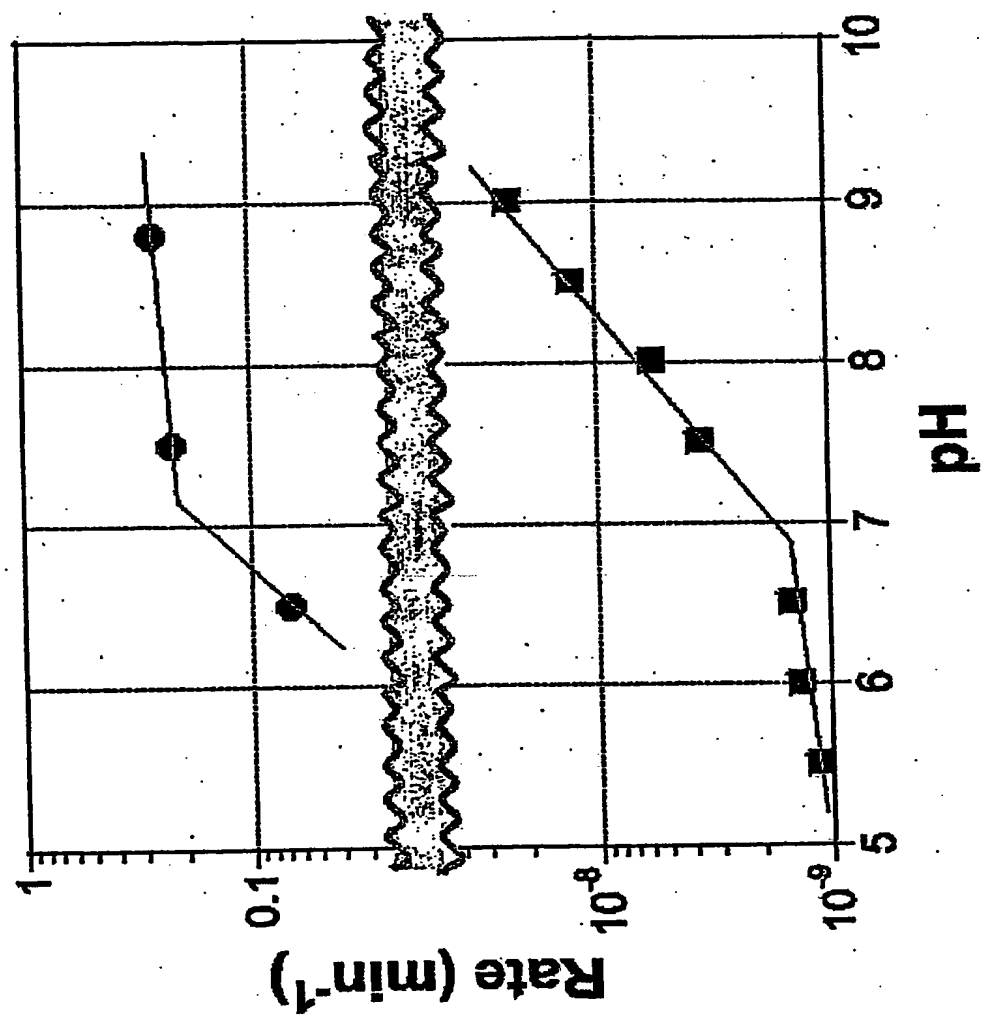
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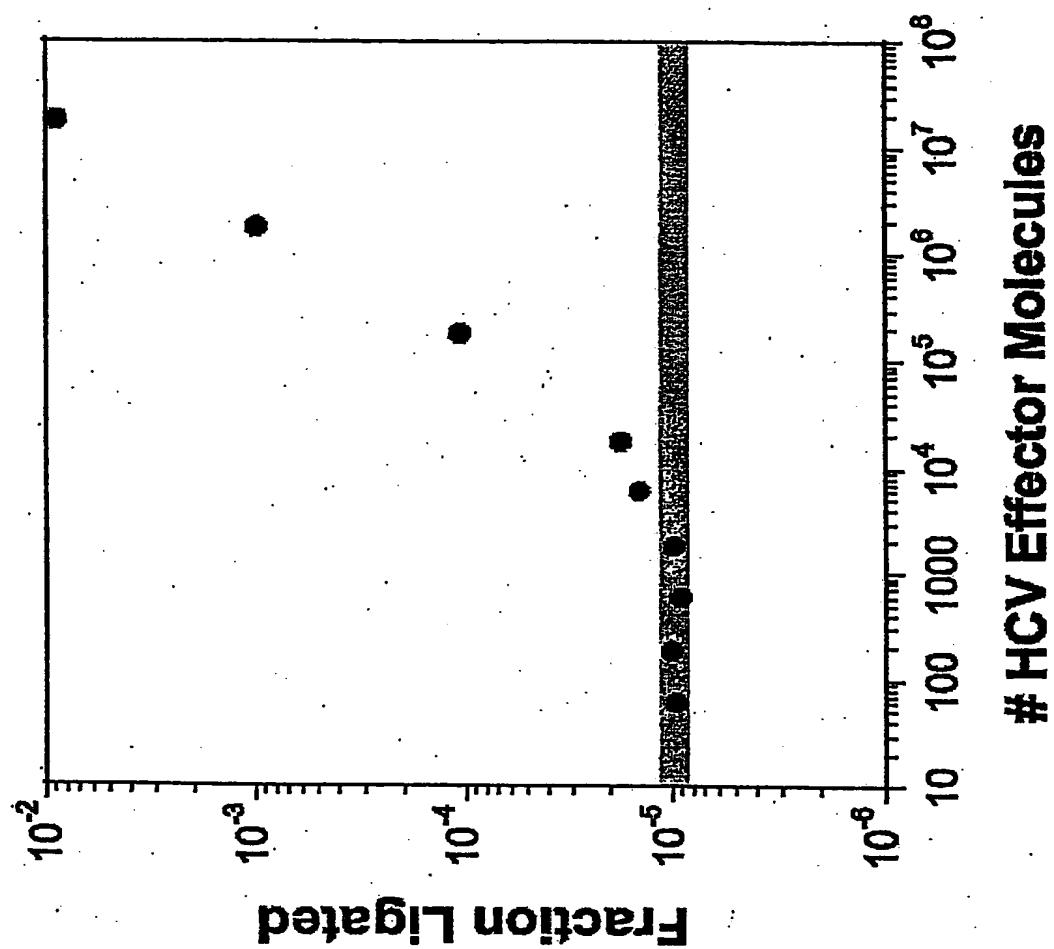
Figure 29

Figure 30

Figure 31

Figure 32

Optimal Clones From DME-1

region 'doped' with mutation frequency of 38%

Optimal Clones From DME-2

Insertion site